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COMPARISON OF CLINICAL MANIFESTATIONS OF RESPIRATORY ILLNESSES DUE TO ASIAN STRAIN INFLUENZA, ADENOVIRUS, AND UNKNOWN CAUSE

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IN A RECENT report, Grieble and associates,[1] have expressed general optimism in regard to the diagnosis of the relatively few specific viral infections which contribute to the common respiratory illnesses seen in a civilian population. The clinical diagnosis of specific respiratory infections in hospitalized military recruits has been considered even more likely because of general awareness of the agents prevalent and the homogeneity of the population. Illnesses which could be proved to be due to adenovirus occurred simultaneously with those related to Asian strain influenza among Naval recruits at the U. S. Naval Training Center, Great Lakes, Illinois, in the summer of 1957. This provided an ideal opportunity for a comparative study of clinical signs and symptoms which could be helpful in the differentiation of illnesses caused by these viruses. The characteristics of illnesses which were not related to either of these agents could also be defined. Previous studies by Hilleman and co-workers[2] have suggested that the simultaneous occurrence of adenovirus and influenza infections among recruits is not unusual. Moreover, the evaluation of influenza vaccines in the past may have been hampered by the inability to differentiate illnesses caused

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by other agents which obscured the effect of the vaccines.[3] The projected use of vaccines against both influenza and adenovirus in recruits throughout the Armed Forces increases the need for information which will make clinical diagnosis useful in the evaluation of the vaccines' effectiveness. Present studies of vaccines rely either on relatively inaccurate hospital admission rates for all respiratory illnesses or extensive and costly laboratory studies. The present study was undertaken to supplement the specificity of modern laboratory diagnosis with clinical data useful to the clinician in the diagnosis of individual cases or large outbreaks of viral illness.

MATERIALS AND METHODS

Patients Studied.—Only recruits who were admitted to a dispensary for inpatient care for respiratory illness were included in this study. All men had an oral temperature of 100° F. or greater at "sick call" prior to admission. Patients were admitted by dispensary medical officers who subsequently managed their case. On the day after admission, each recruit was seen independently by a medical officer of the Naval Medical Research Unit No. 4 who obtained a history according to a standard questionnaire, completed a brief physical examination, and recorded all findings with a diagnosis which reflected the general anatomic area of predominant involvement. At this time, a throat swab was taken from all patients seen. In addition, a blood specimen was drawn from all men who would be available for a second specimen during convalescence. Clinical data were analyzed from the study questionnaire initially, and later, charts and x-rays were also reviewed. Certain commonly studied aspects of illness were not analyzed in detail, i. e., duration of hospitalization, degree of fever, and white blood counts. Clinical data are representative of the status 24 hours after admission and at varying intervals from onset of symptoms in individual cases. Such data are comparable to the kind of information available to the admitting physician who determines diagnosis and treatment of patients.

An evaluation of influenza and adenovirus vaccines was in progress from July, 1957, to March, 1958, and includes some of the men in this report. The vaccine administered was only taken into consideration when an attempt was made to differentiate a serologic response to the vaccine and a bona fide infection. Because of the degree of protection afforded by vaccines,[4] a majority of the patients to be reported on were men who had not received either vaccine. Elevated antibody titers produced by vaccines can sometimes make serologic diagnosis difficult.[5] Therefore, the group of patients referred to as the "unknown group" was arbitrarily confined to men who received neither influenza nor adenovirus vaccine. Because seasonal allergic symptoms might be confused with respiratory disease, subjects in whom there was a specific personal or strong family history of allergy were not included in the analysis. Any further selection of patients for study was determined only by the results of laboratory tests on available specimens.

Viral Isolation.—Standard methods for the isolation and identification of viruses have been described in detail elsewhere.[6, 7] Throat swabs were immediately placed in prepared screw-capped vials containing gelatin-lactalbumin yeast extract (GLY) maintenance media,[8] and stored at −65° C. until used. Because of the need to conserve laboratory facilities, only a limited number of specimens collected were analyzed. Attempts at virus isolation were made on specimens drawn from the freezer in a random fashion. Influenza viruses were isolated in embryonated chicken eggs and typed by the hemagglutination inhibition test by the method of Hirst as modified by Salk[9] using specific rooster antiserum against A'/JAPAN/305, the prototype for all Asian strains isolated. Adenovirus and Coxsackie viruses were isolated in HeLa cell cultures* and were typed with the use of specific rabbit antisera. HeLa cells

*Stock cultures and Eagle's basal media were obtained from Microbiological Associates, Inc., Bethesda, Md.

were inoculated with 0.2 ml. of the specimen and were observed on alternate days. At the end of 2 weeks the cultures were passed to new HeLa cells and observed for an additional 2 weeks before they were reported negative.

Complement Fixation Test.—Fifty milliliters of blood obtained by venipuncture was processed to provide serum for a modified Kolmer[10] complement fixation test with the use of influenza and adenovirus antigens standardized by the optimal proportions method of Friedewald.[11] A fourfold or greater rise in titer between the acute and convalescent serum was considered to warrant a diagnosis of infection. Antistreptolysin O determinations were made by the method previously described[12]; commercially available reagents* were used.

Clinical Material.—Of 600 men examined, data sufficient to make a specific etiologic diagnosis or to exclude the diagnosis of influenza, adenovirus, and streptococcal infection were available from 233.' The distribution of illnesses according to etiologic laboratory diagnosis is shown in Table I.

TABLE I. DISTRIBUTION OF CASES ACCORDING TO LABORATORY DIAGNOSIS IN RECRUITS HOSPITALIZED WITH RESPIRATORY ILLNESS (JULY TO OCTOBER, 1957)

GROUP	DIAGNOSIS	NO.	TOTAL
Group I	Influenza		112
	A' Asian virus isolated	14	
	CF* only	98	
Group II	Adenovirus		69
	Type 4 isolated	45	
	CF only	19	
	Type 7 isolated	5	
Group III	Dual infection		
	Influenza and adenovirus, CF		10
Group IV	Unknown (influenza, adenovirus, and streptococcus ruled out)		34
Group V	Coxsackie		7
	B-2 isolated	2	
	B-3 isolated	5	
Group VI	Unidentified cytopathogenic agent		1
			233

*CF = complement fixation test.

Since all available paired sera were examined by the complement fixation test, there are cases in which the diagnosis was made only by this test, either because the throat swab was negative or not tested. Four of the patients from whom Asian strain influenza virus was isolated and 37 of 45 patients from whom adenovirus Type 4 was isolated were diagnosed by the complement fixation test. These represent the only cases for which serum specimens were available. In the subsequent comparison of cases, the 3 major groups, influenza, adenovirus, and unknown, are analyzed without regard to the particular method of laboratory diagnosis. The adenovirus group analyzed does not include the 5 patients from whom Type 7 was isolated because there have been reports which suggest a distinct clinical illness characteristic of Type 7 as compared to Type 4 infection.[13]

The over-all characteristics of respiratory illness during the study period have been reported elsewhere.[4] It appeared that a gradually increasing incidence of Asian strain influenza was superimposed on a steady rate of adenovirus infection and other illnesses which are endemic at this Training Center throughout the year. The distribution of cases by month is shown in Table II. It is evident that adenovirus and influenza were both prevalent throughout the period of observation and dual infections were detected serologically in 10 men. The cases of dual infection are not considered in detail here but are further evidence

*Purchased from Difco Co., Detroit, Mich.

of the close association of the 2 agents in this population. Although the study was undertaken during the summer months, the role of streptococcal infection deserves consideration. Previous experience has shown that there is considerable difficulty in isolating group A streptococci from men who are admitted to the dispensary with respiratory illness because they have often received antibiotic therapy at sick call with outpatient care prior to admission. We have, therefore, relied more upon carrier rates as determined by a random sampling of men in training and those who report to sick call with untreated illness in determining the prevalence of streptococcal infection. When the streptococcal carrier rate remains low (1 to 4 per cent), it has been unusual to detect any significant number of illnesses that could be attributed to streptococci by analyses of sera for a rise in antistreptolysin O titer. The carrier rates (based on total cultures taken) in July, August, and September of 1957 were 1.7 per cent, 1.1 per cent, and 3.9 per cent, respectively. Thirty-four men in the unknown group who did not show a serologic response to adenovirus or influenza also failed to show a rise in antistreptolysin O titer. This is evidence that the etiologic role of streptococci was minimal.

TABLE II. DISTRIBUTION OF CASES OF RESPIRATORY ILLNESS IN MAJOR ETIOLOGIC GROUPS BY MONTH OF OBSERVATION

TIME (MONTH AND DAYS)	ETIOLOGIC DIAGNOSIS			
	GROUP I INFLUENZA	GROUP II ADENOVIRUS	GROUP III DUAL	GROUP IV UNKNOWN
July 15-31	2	30	—	15
Aug. 1-31	75	27	7	12
Sept. 1-30	33	9	3	6
Oct. 1-5	2	3	—	1
Total	112	69	10	34

TABLE III. CHIEF COMPLAINT*

COMPLAINT	ETIOLOGIC GROUP					
	INFLUENZA		ADENOVIRUS		UNKNOWN	
	NO.	%	NO.	%	NO.	%
Headache†	39	34.8	5	7.8	6	17.7
Sore throat†	14	12.5	25	39.1	8	23.5
Fever and/or chills	31	27.7	18	28.1	6	17.7
Cough	8	7.1	8	12.5	5	14.7
Weakness/tiredness	7	6.2	4	6.3	4	11.8
Chest pain	0	0.0	3	4.7	2	5.9
Gastrointestinal	7	6.3	0	0.0	2	5.8
Other	6	5.4	1	1.6	0	0.0
Total	112		64		34	

*The incidence of various chief complaints in each of the 3 etiologic groups of cases is shown. Data which are statistically significant ($p < 0.05$) in comparing any of the 3 categories are at the top of the table and are marked with a dagger (†).

RESULTS

Onset.—Sudden onset was arbitrarily defined as hospitalization within 24 hours after the first symptom. All other types of onset were considered to be gradual. There was a significantly higher incidence of sudden onset in the influenza group (61.6 per cent) compared to both adenovirus (35.9 per cent) and unknown (38.2 per cent) groups. The subjective responses of the men who were proved to have influenza also reflected this; many were able to localize onset to the hour, and were hospitalized promptly.

Chief Complaints.—The chief complaint was elicited in a manner which would provide information about the single symptom which was most responsible

for the recruit's reporting to the infirmary. Accordingly, men were asked, "What bothered you most on the day you were admitted?" The first symptom mentioned was recorded as the chief complaint. The data are summarized in Table III.

Feverishness, sore throat, weakness, and cough were the most common chief complaints. Fever and weakness were represented with about equal frequency in all groups, and differences in the incidence of cough are not statistically significant. Headache and sore throat as chief complaints appeared useful in the differentiation of illnesses caused by adenovirus and influenza. The relative incidence of these symptoms in the 2 groups was highly significant ($p < 0.01$) with headache as chief complaint in 34.8 per cent of influenza cases and only 7.8 per cent in adenovirus cases. Sore throat was the chief complaint in 39.1 per cent of adenovirus cases and only 12.8 per cent of influenza cases. Headache was significantly increased in the group with influenza when compared to the unknown group as well. In other respects, the unknown group appears to occupy an intermediate position between the 2 specific diagnostic groups.

TABLE IV. SYMPTOMS*

SYMPTOM	ETIOLOGIC GROUP					
	INFLUENZA		ADENOVIRUS		UNKNOWN	
	NO.	%	NO.	%	NO.	%
Headache†	78	69.6	35	54.7	24	70.6
Dysphagia†	43	38.4	47	73.4	17	50.0
Hoarseness†	31	27.7	38	59.4	13	38.2
Chest pain†	17	15.2	10	15.6	11	32.4
Nonproductive cough†	63	56.3	28	43.8	12	35.3
Sore throat, total†	69	61.6	53	82.8	22	64.7
Pharyngeal†	43	38.4	40	62.5	10	29.4
Laryngeal	23	20.5	10	15.6	7	20.6
Tracheal	9	8.0	8	12.5	3	8.8
Fever and/or chills	108	96.4	60	93.8	28	82.4
Productive cough	36	32.1	26	40.6	15	44.1
Nasal symptoms	64	57.1	36	56.3	19	55.9
Conjunctival symptoms	28	25.0	22	34.4	8	23.5
Malaise	77	68.8	39	60.9	24	70.6
Gastrointestinal	24	21.5	14	21.9	7	20.6
Eye pain	5	4.5	0	0.0	1	2.9
Myalgia, arthralgia	40	35.7	17	26.5	9	26.5
Other	5	4.5	3	4.7	3	8.7
Total number of patients	112		64		34	

*The incidence of various symptoms in each of the 3 etiologic groups of cases is shown. Data which are statistically significant ($p < 0.05$) in comparing any of the 3 categories are at the top of the table and are marked with a dagger (†).

Symptoms.—Table IV indicates the incidence of symptoms for each of the diagnostic groups as obtained in the interviews with patients. As expected from the general criteria for admission, fever and chills, which were so intimately linked as to be considered inseparable, were prominent in all groups although slightly less so in the unknown group. Multiple symptomatology was quite common and was encouraged by the examiner's technique which required a specific response to each symptom listed.

Headache and sore throat are important in the differentiation of illnesses of different etiology. Headache, present in 69.6 per cent of patients with illness

caused by influenza virus, was significantly increased as compared to 54 per cent in the adenovirus group. Although 70 per cent of the patients in the unknown group also complained of headache, these data lack statistical significance. Sore throat, on the other hand, was significantly more frequent ($p < 0.01$) in the adenovirus group (82.8 per cent) compared to both influenza (61.6 per cent), and unknown (67.7 per cent) groups. Moreover, localization of sore throats to the pharynx was significantly more prevalent in the adenovirus group. Similar data were obtained regarding the symptoms of dysphagia and hoarseness. Nonproductive cough was significantly more frequent in the influenza group (56.3 per cent) only when compared with the unknown group, 35.3 per cent. On the other hand, chest pain was present in 32.4 per cent of the unknown group and was less frequent in both influenza and adenovirus groups.

TABLE V. PHYSICAL FINDINGS*

FINDING	ETIOLOGIC GROUP					
	INFLUENZA		ADENOVIRUS		UNKNOWN	
	NO.	%	NO.	%	NO.	%
Lymphoid hyperplasia†	51	45.5	42	65.6	16	47.1
Pharyngitis†	33	29.5	33	51.6	9	26.5
Exudate†	6	7.1	14	21.9	2	5.9
Negative for all other	27	24.1	9	14.1	4	11.8
Lung signs	28	25.0	18	28.1	12	35.3
Conjunctivitis	17	15.1	8	12.5	4	11.8
Cervical nodes	15	13.4	4	6.3	4	11.7
Rhinitis	10	8.9	12	18.8	5	14.7
Post nasal drip	4	3.6	5	7.8	3	8.8
Tonsilitis	9	8.0	6	9.4	4	11.8
Exudate	4	3.6	5	7.8	4	11.8
Total number of patients	112		64		34	

*The incidence of various physical findings in each of the 3 etiologic groups of cases is shown. Data which are statistically significant ($p < 0.05$) in comparing any of the 3 categories are at the top of the table and are marked with a dagger (†).

Physical Findings.—Comparative data on physical findings observed in each of the 3 groups are shown in Table V. Only physical examination of the throat was of significance in differentiating the adenovirus group and the other 2 groups. The increased incidence of pharyngeal lymphoid hyperplasia (65.6 per cent), injection (51.6 per cent), and exudate (21.9 per cent) in patients with adenovirus was highly significant compared to those with influenza. Pharyngeal injection and exudate were similarily more common in patients with adenovirus than in the unknown group, but the data concerning lymphoid hyperplasia were not statistically different. Exudate varied from large patches to small punctate areas. A true punctate lesion was seen only rarely, however, in this series and may represent an early stage of pharyngeal inflammation.

Final Diagnosis.—The examiner's final diagnosis, made prior to laboratory reports, is shown in Table VI. As expected, the diagnosis is a reflection of the significant data in the history and physical examination which distinguished the cases with different etiologies. Thus, pharyngitis as a final diagnosis representing the predominant anatomic site of involvement was significantly more frequent in the adenovirus group (51.6 per cent) than in the influenza or unknown groups. Diagnosis of "systemic infection" was used for those patients in which

no localized area of involvement could be found and represents patients in which constitutional and systemic symptoms were predominant. This diagnosis was made more frequently in cases later proved to be due to influenza (31.3 per cent) than in cases diagnosed as adenovirus infections or cases of unknown etiology.

Diagnosis of Pneumonitis.—As indicated earlier, results of chest x-rays were only noted after completion of the study and were not available to the examiners at any time. Table VII summarizes the diagnosis of pneumonitis in which x-ray and clinical information were utilized. It must be emphasized that chest x-rays were made only of patients selected by Ward Medical Officers and, as indicated, the number of patients selected varied with the diagnostic groups. For reasons which are not clear, a greater percentage of cases that were due to adenovirus

TABLE VI. EXAMINER'S DIAGNOSIS*

	ETIOLOGIC GROUP					
	INFLUENZA		ADENOVIRUS		UNKNOWN	
DIAGNOSIS	NO.	%	NO.	%	NO.	%
Pharyngitis†	32	28.6	33	51.6	8	23.5
Systemic infection†	35	31.3	8	12.5	7	20.6
Pneumonia	5	4.5	2	3.1	6	17.7
Bronchitis	34	30.4	16	25.0	10	29.4
Tonsilitis	6	5.4	5	7.8	3	8.8
Total	112	100.0	64	100.0	34	100.0

*The incidence of various diagnoses in each of the 3 etiologic groups of cases is shown. Data which are statistically significant ($p < 0.05$) in comparing any of the 3 categories are at the top of the table and marked with a dagger (†).

TABLE VII. DIAGNOSIS OF PNEUMONITIS

	ETIOLOGIC GROUP		
METHOD OF DIAGNOSIS	INFLUENZA	ADENOVIRUS	UNKNOWN
By x-ray (No. cases)	9	7	4
Per cent of total cases x-rayed	30.4	53.1	32.4
Per cent positive of those x-rayed	26.5	20.6	36.4
By examiner (No. cases)	1	0	2
Total pneumonitis (No. cases)	10	7	6
Per cent of total cases	8.9	10.9	17.6

were selected for x-ray study at some time in the course of their illness. Seventeen of the 20 cases diagnosed by x-ray were diagnosed clinically as either bronchitis or pneumonitis.

An earlier report[14] suggested the incidence of pneumonitis was approximately 10 per cent in all groups, but the adoption of more rigid criteria for the unknown group altered the results somewhat and the present series reveal a higher incidence of pneumonitis in this group. All of the patients with pneumonia were studied in detail; the study included hospital and laboratory records. Sputum cultures obtained prior to the initiation of antibiotic therapy ruled out bacterial pneumonitis in every instance. There were no fatalities in the entire series, and no patients were critically ill at any time.

Correlation of Clinical and Laboratory Diagnosis.—Fig. 1 shows all data which were significantly different ($p < 0.05$) in comparing the 3 etiologic groups. Although there are numerous signs and symptoms which appear with

significantly greater frequency in each disease group, the usefulness of these data is limited by the areas in which etiologic groups tend to overlap. To estimate the degree of this overlap, the examiner's diagnosis, which was in essence the result of significant differences in the history or physical examinations, was analyzed. The diagnoses were divided into 3 broad categories, upper respiratory, systemic, and lower respiratory infection (URI, systemic, and LRI), based on the anatomic area of predominant clinical involvement. The pattern of illness in this mixed epidemic is presented in Fig. 2 and is based on the assumption that the series described here was representative of the entire respiratory illness experience in the period studied. This assumption cannot be made without hesitation because of possible bias in case selection indicated earlier.

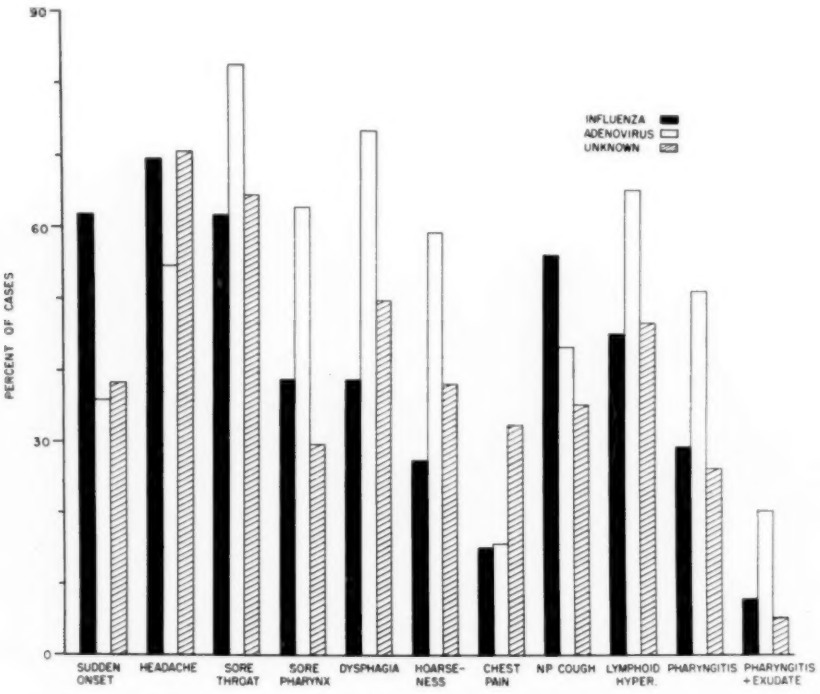

Fig. 1.—Summary of data which are significant in comparing illness caused by influenza, adenovirus, or of unknown etiology. The incidence of findings is indicated for each etiologic group whenever differences between any 2 of the groups is statistically significant ($p < 0.05$).

As indicated in Fig. 2, the most useful diagnosis associated with specific illness was systemic infection since influenza accounted for 70 per cent of cases with this clinical diagnosis. Although most of the adenovirus cases were diagnosed URI, as can be seen from the relative size of the areas depicting adenovirus illness in Fig. 2, an equal number of cases with this diagnosis were due to influenza, limiting the usefulness of this diagnosis in this series. A majority of LRI were caused by influenza, but the comparative analysis (Table VI) did not indicate a closer association with influenza compared to the other agents. Much of the collateral data of this report were, of course, not available to the

examiners in arriving at a simple anatomic diagnosis, so that the ultimate usefulness of this information will depend on its application in unselected cases where an attempt is made to weigh all factors and make a specific diagnosis by clinical methods.

DISCUSSION

The preceding results can be used to delineate a composite clinical picture representative of a majority of cases in each etiologic group. Fever and chills were prominent in all groups. Asian strain influenza was characterized by sudden onset, headache (often the chief complaint), malaise, sore throat (pharyngeal or laryngeal in location), nonproductive cough, nasal symptoms, and nonlocalizing physical findings. Type 4 adenovirus infection was characterized by gradual onset, sore throat (often the chief complaint and usually pharyngeal in location), malaise, dysphagia, hoarseness, nasal symptoms, headache, and findings of pharyngeal inflammation with or without exudate. The

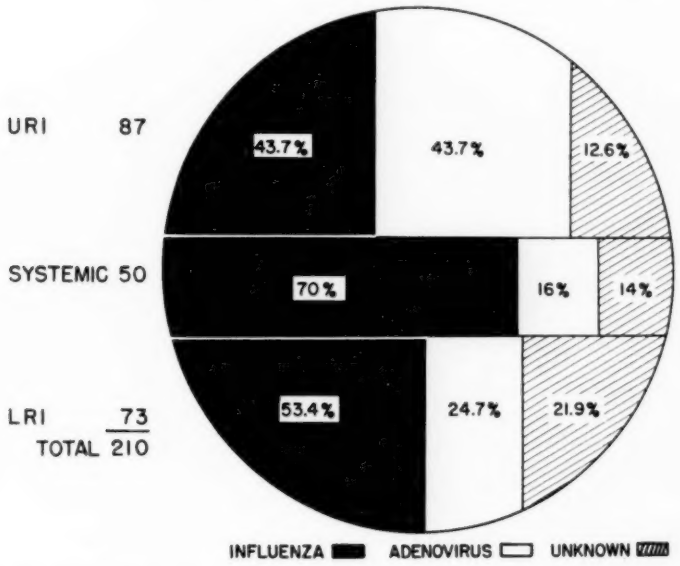

Fig. 2.—Comparison of clinical and etiologic diagnoses. The areas of the diagram represent the per cent of cases in each diagnostic category which were due to the etiologic agents indicated.

majority of cases in the unknown group tended to resemble the influenza cases except for gradual onset and minor differences in less common signs and symptoms.

These studies indicate the subtle, but real differences in the composite clinical picture of illness caused by influenza and adenovirus infections. This type of dichotomy was recognized many years ago in studies by Stuart-Harris[15] who outlined the difference between clinical influenza and the associated "febrile catarrhs" which have been described under different names and more recently associated with the adenoviruses.[2] Only with the discovery of the adenoviruses

and their association with specific illness in subsequent clinical and epidemiologic studies[2, 6, 13, 16] could the definitive characteristic of the illness associated with these agents be compared directly to cases of proven influenza as has been done in this study. There are observations which might have aided in the clinical diagnoses that were not developed in this study. Among these are the duration of illnesses and their course which were not evaluated. In addition, temperature curves and such routine laboratory data as leukocyte counts might have been helpful. In spite of these limitations, there was sufficient data available to encourage those who would seek clinical definition of specific viral respiratory illness.

In the winter of 1952 to 1953, Hilleman and co-workers[2] observed an outbreak of respiratory illness caused by a new agent, RI-67. This agent was subsequently classified as adenovirus Type 4 and associated not infrequently with respiratory illness in many military installations.[17] In describing their experience with this new agent, Hilleman and associates indicated the simultaneous occurrence of cases of A′ influenza; but the clinical analysis of cases was confined only to those related to the new virus. Originally isolated from a recruit with primary atypical pneumonia, RI-67 was, nevertheless, associated with a clinical illness quite similar to that described in the present study. The discovery of this agent helped to provide a specific etiology for illness referred to as acute respiratory disease (ARD).[18, 19]

Subsequent studies have confirmed the relationship of adenoviruses to much of the acute respiratory disease seen in hospitalized military recruits. In Fort Ord, California, Berge and associates[13] described a series of 51 cases in which Types 3, 4, and 7 were isolated. The authors suggested that Type 7 was usually associated with upper respiratory and Type 4 with lower respiratory illness, but their data do not support so strong a conclusion.

In detailed clinical studies of 45 hospitalized patients with proved adenovirus infection, principally Type 7, Dascomb and Hilleman[20] described a basic syndrome of gradual onset, fever, pharyngitis, and cough often accompanied by either symptoms of bronchitis, conjunctivitis, or otitis. All of their patients had abnormal throat findings, some with exudate which varied from discrete pinpoint lesions to large confluent patches. A study of Naval recruits at San Diego revealed prominent signs and symptoms of pharyngeal inflammation in cases diagnosed by complement fixation tests for adenovirus.[21] Human volunteers, injected intramuscularly with live pooled virus Types 3, 4, and 7 became ill with fever, inflammation of the eyes, ears, nose, and throat with prominent lymphoid hyperplasia.[22]

There have been many studies of the clinical representation of influenza since the isolation of the A virus in 1933.[23] Despite changes in the strains isolated, the clinical descriptions have deviated little from the classical descriptions by Stuart-Harris.[15] In a clinical study of influenza A′ infection at Fort Monmouth, Kilbourne and Loge[24] were able to delineate a characteristic clinical syndrome which was useful in the differentiation of influenza and streptococcal illness. They described A′ influenza as primarily an upper respiratory illness

associated with constitutional symptoms, but reported a very low incidence of lower respiratory involvement. As in our cases, the sore throat of influenza was rarely dysphagic and the cough was usually nonproductive. Subsequently, an A′ epidemic analyzed at the U. S. Naval Hospital, Philadelphia, by Scher and Jaruszewski[25] established the occurrence of pneumonitis related specifically to viral infections without implicating superinfection with other organisms. With exception of findings related to the lungs, cases with and without infiltration presented similar clinical findings. Sudden onset has been recognized as pathognomonic of influenza in all series.

In recent reports on the clinical representation of Asian influenza, there has been little to suggest any deviation from classical descriptions recognized for many years.[26] Some authors who analyze clinical data on large numbers of cases are content to establish the etiologic diagnosis in a few typical cases and ascribe all other respiratory illness which occur in the same period to the same agent. At the 1957 Boy Scout Jamboree,[27] 616 cases were analyzed based on a specific diagnosis in 17 of 31 selected cases for laboratory study. In another large series, our laboratories aided in the identification of the causative agent in 16 of 682 cases reported by Greene and Hair[28] from the U. S. Naval Hospital in Memphis, Tennessee. Among their cases, the most significant symptoms were headache, fatigue, nonproductive cough, chills, and ocular pain. The latter symptom was not prominent in our series. The occurrence of bronchitis and bronchopneumonia was infrequent and was considered a complication of Asian influenza by Greene and Hair. Our data suggest that these findings are part of the presenting clinical manifestations of the infection in some of our patients.

In the small selected series of cases which we have called "unknown," there are reflected some of the elements of difficulty in establishing an etiologic diagnosis in every case of respiratory illness even during epidemics. We are unable to accept the contention that these are merely serologically negative cases of either adenovirus or influenza despite some clinical resemblance to the latter. We prefer to consider these cases of heterogenous etiology. Preliminary results of complement fixation tests for Coxsackie virus, which are still in progress, suggest that some of the cases are causally related to this agent. There is no evidence that the individuals from whom these sera were obtained were unable to respond to antigenic stimuli in the conventional manner.

The data of this report re-emphasize the importance of clinical observations in the diagnosis of viral respiratory infections. The diagnosis of outbreaks of specific illness remains the clinician's responsibility and is not difficult. Shifts in the incidence of specific diseases can readily be detected, particularly among military recruits who are heavily infected. Large field trials of vaccine effectiveness may have to rely heavily upon clinical data since laboratory results are necessarily limited.

SUMMARY

1. Clinical manifestations of respiratory illness caused by Asian strain influenza, Type 4 adenovirus, and unknown cause were studied among 210 hospitalized Navy recruits during the summer of 1957.

2. Significant differences were observed in the composite clinical picture associated with 112 cases of influenza and 64 cases of adenovirus infection. Sudden onset and headache were more prominent in illnesses caused by influenza virus, while pharyngeal sore throat, dysphagia, hoarseness, and physical findings in the throat were more prominent in illnesses caused by adenovirus.

3. Asian strain influenza was characterized by sudden onset, fever, headache (often the chief complaint), malaise, sore throat (pharyngeal or laryngeal in location), nonproductive cough, nasal symptoms, and nonlocalizing physical findings.

4. Type 4 adenovirus infection was characterized by gradual onset, fever, sore throat (often the chief complaint and usually pharyngeal in location), malaise, dysphagia, hoarseness, nasal symptoms, headache, and findings of pharyngeal inflammation with or without exudate.

5. Pneumonia which appeared to be a part of the viral infection was diagnosed by x-ray or clinical examination in 8.9 per cent of influenza and 10.9 per cent of adenovirus infections studied.

6. Cases presenting with symptoms of systemic infection with few or no physical findings were most readily diagnosed as influenza.

7. Thirty-four cases in the unknown group, in which influenza, adenovirus, and group A streptococci had been ruled out, resembled influenza clinically but manifested a higher incidence of pneumonia. These cases appeared to have diverse etiologies.

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INVALIDATION OF THE HYPOTHESIS OF DYSPROTEINEMIA AS A CAUSE OF PROTEINURIA IN EXPERIMENTAL NEPHROSIS

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NORMAL urine long has been assumed to be protein-free, and the presence of protein in the urine has been considered indicative of renal disease. The demonstration of absence of protein from the glomerular filtrate in frogs by Wearn and Richards[1] strengthened these assumptions. However, the idea that normal urine is protein-free has been challenged by many investigators. Mörner[2] demonstrated the presence of 22 to 78 mg. of protein per liter of urine in humans. Gunton and Burton[3] found the mean concentration to be 3.7 mg. per 100 ml. The mean value for urinary output of protein per 24 hours was found by Rigas and Heller[4] to be 39 mg. (range 30.5 to 49.6 mg.).

After producing renal damage in fish, Bieter[5] found that proteinuria developed in those whose kidneys had glomeruli but not in those whose kidneys were aglomerular. Addis[6] administered serum from rats to others of the same strain and produced proteinuria without renal damage. He attributed the proteinuria to increased concentration of proteins in the plasma. Similar findings were obtained in dogs given dog plasma[7] and in patients with cirrhosis of the liver after several intravenous injections of human serum albumin.[8] These various findings provided support for the concept that glomerular filtration is the mechanism for the appearance of protein in the urine. In their study of the mechanism of proteinuria in patients with the nephrotic syndrome, Chinard and co-workers[9] concluded that proteinuria occurs as a result of increased permeability of the glomerular capillary walls to protein rather than solely as the result of decrease in reabsorption of protein by the tubules.

The mechanism of proteinuria has been the subject of several hypotheses, prominent among which are the following:

1. Proteinuria is produced by damage to the glomerular membranes that increases their permeability and leads to leakage of proteins from the plasma through the glomerular wall with the glomerular filtrate into the tubular lumen. Abnormal glomerular capillaries leaked plasma proteins, but the tubules were considered absolutely passive in that matter.

2. In normaley the tubular epithelium completely reabsorbs the minute amounts of plasma proteins that find their way into the tubular lumen, but diseased glomerular capillaries leak excess plasma proteins far beyond the

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maximal absorptive capacity of the tubular parenchyma to cope with the leakage, and proteinuria results.

3. The renal tubular epithelium secretes proteins into the urine.

4. Alteration of plasma proteins (dysproteinemia) permits their passage through the glomerular membranes.

The purpose of this study is to assess this last concept.

METHODS

Production of Nephrosis in Rats.—Experimental nephrosis was produced in adult albino rats by intravenous administration of serum obtained from rabbits sensitized against rat kidney.

These rabbits had been given intraperitoneally rat kidney homogenate prepared as follows. By aseptic technique the kidneys were removed from rats, sliced into very fine pieces, washed several times with sterile saline (0.9 per cent) until the saline remained clear and the renal tissue became free of blood. To the washed and drained renal tissue, an equal weight of saline was added; and the mixture was homogenized in a sterile blender (Waring Blendor). This made a 50 per cent kidney homogenate, to which 5 ml. of toluene and 1,000 U. of aqueous penicillin per milliliter of homogenate were added; the mixture was incubated for 24 hours at 37° C.

The sterile homogenate was diluted with saline to 20 per cent and was injected intraperitoneally into rabbits once every second day: 5 ml. at the first injection, 7.5 ml. at the second, and 10 ml. at each of 10 injections afterward. One week after the last injection the rabbits were exsanguinated under aseptic precautions by cardiac puncture, and the serum was separated by centrifugation.

Litter mates from albino rat colonies weighing 250 to 300 grams were prepared. With suprapubic cystotomy a mushroom polyethylene tube was placed in the urinary bladder as an indwelling catheter for continuous collection of the urine. The urine ran freely through the catheter into a flask. A fine polyethylene tube was anchored in the tail vein for intravenous administration of the nephrotoxic serum. Each rat was placed in a separate cage; food and water were available ad libitum. The rabbit serum was administered intravenously in amounts of 0.25 ml. or more every hour into the tail vein. Up to 10 ml. was given over 3 days.

From the second day after the start of the administration of nephrotoxic serum, an increase in leakage of plasma proteins into the urine was observed. The peak of hypoproteinemia, hyperlipoidemia, and proteinuria usually occurred about the sixth day.

Transfers of Blood and Urine.—At the peak of the nephrosis, as complete an exchange transfusion as possible was made between the nephrotic rats and their normal litter mates. Blood was withdrawn by cardiac puncture from a rat into a syringe containing 1 ml. of 2.5 per cent sodium citrate, and was administered into the tail vein of another. About 10 ml. of blood was taken from each rat and exchanged for a similar amount from its litter mate.

Another group of rats similarly prepared and placed in individual cages was given intravenously through the tail vein Ringer's solution which contained the proteins extracted from the urine of the nephrotic rats. The concentration was 10 to 15 Gm. of urinary protein per 100 ml. of Ringer's solution. Each rat received during a 10 hour period from 0.5 to 1.5 Gm. of the proteins obtained from the urine of its nephrotic litter mates.

Protein Patterns.—Before and at intervals after the exchange transfusion, the patterns of urinary and plasma proteins of the nephrotic and of the normal rats were determined electrophoretically, and the total proteins were quantitated chemically.

In another study experimental nephrosis was similarly produced in dogs. Among other determinations the urinary and serum proteins were analyzed electrophoretically by both paper chromatography and the Tiselius procedure. The protein fractions found in the urine were practically identical with those in the serum of the same animal (Fig. 1).

RESULTS

Normal rats eliminate a certain amount of protein in the urine. Consequently it is not the presence or absence of protein in the urine that must be considered, but the relation between the amounts of urinary excretion of protein established during the control period and the corresponding amounts quantitated in the period which follows the exchange transfusions and the injections of urinary proteins.

TABLE I. URINARY PROTEINS (GRAM PER 100 ML.) FROM NORMAL RATS BEFORE AND AFTER TRANSFUSION OF BLOOD FROM NEPHROTIC LITTER MATES

RAT NO.	BEFORE	AFTER
1	0.47	0.28
2	0.38	0.28
3	0.13	0.09
4	0.27	0.08
5	0.49	0.16
6	0.25	0.04
7	0.15	0.16
8	0.14	0.06
9	0.30	0.15
10	0.11	0.13
11	0.11	0.13

TABLE II. URINARY PROTEINS (GRAM PER 100 ML.) FROM NORMAL RATS BEFORE AND AFTER INTRAVENOUS INJECTION OF URINARY PROTEINS FROM NEPHROTIC LITTER MATES

RAT NO.	BEFORE	AFTER
12	0.15	0.15
13	0.23	0.08
14	0.13	0.11
15	0.21	0.22
16	0.26	0.27

Normal Rats Given Blood From Nephrotic Rats.—The amounts of urinary proteins in the urine of 11 normal rats before and after they received blood from their nephrotic litter mates (practically complete exchange transfusion) are shown in Table I. It is evident that in the control period all 11 rats excreted protein in their urine, the amounts varying between 0.49 and 0.11 Gm. per 100 ml. After the exchange transfusion, however, they responded by a general decrease in urinary excretion of protein. Three rats showed an insignificant increase (0.01 and 0.02 Gm. per 100 ml.), and the other 8 showed definite reduction.

Transfusion of blood from nephrotic rats into normal rats did not significantly affect the proteinuria present before the transfusion. If the plasma proteins in the nephrotic rats had been altered, and if the alteration had facilitated their passage across the glomerular capillary membranes, the normal rats that received the blood from the nephrotic rats which contained these supposedly altered proteins should have lost them into the urine and caused an increase in the quantity of proteins measured therein.

Normal Rats Receiving Urinary Proteins From Nephrotic Rats.—The quantities of protein found in the urine of 5 normal rats before and after they received intravenously in Ringer's solution the extracted proteins from the

urine of nephrotic litter mates are given in Table II. Of these 5 rats 1 showed no change in the quantity of urinary proteins, 2 showed reductions, and 2 showed insignificant increases.

If the proteins extracted from the urine of nephrotic rats had leaked into the urine because of alteration in their structure, they should have likewise leaked into the urine of the normal rats which received them intravenously and should have caused a marked increase in their urinary proteins.

Nephrotic Rats Given Blood From Normal Rats.—The concentrations of urinary proteins of 5 nephrotic rats before and after they received blood from normal litter mates in exchange for their blood are shown in Table III. From their previously elevated levels, the amounts of urinary proteins were increased in 2, were much reduced in 2, and were slightly reduced in 1.

TABLE III. URINARY PROTEINS (GRAMS PER 100 ML.) FROM NEPHROTIC RATS BEFORE AND AFTER TRANSFUSION OF BLOOD FROM NORMAL LITTER MATES

RAT NO.	BEFORE	AFTER
17	4.20	3.56
18	3.41	3.81
19	3.60	1.96
20	4.98	1.51
21	3.37	4.28

These variations in the urinary proteins of the nephrotic rats which received blood from normal rats are attributable to the degree of involvement of the kidney by the nephrotic process. In those that showed increased loss of proteins into the urine, the involvement of the kidneys with the nephrotic process was found to be more extensive than in those that showed reduction. Evidently the administration of normal blood which contained the normal plasma proteins provided more proteins for the diseased kidneys to leak.

Tables I and II present evidence against alteration of plasma proteins, and Table III gives supportive evidence for the suggestion that damage (as by renal lesions) to the glomerular membranes, with resultant increased permeability, is the basic cause for the increased loss of plasma proteins into the urine.

Similarity of Urinary and Plasma Proteins.—Fig. 1 presents, by both the paper chromatographic and the Tiselius procedures, the electrophoretic patterns of the urinary and plasma protein fractions. It demonstrates the presence of all the components of the plasma proteins in the urine of nephrotic animals. This is convincing evidence that the plasma proteins are the main source of urinary proteins.

DISCUSSION: SOURCE AND INGRESS OF PROTEIN IN URINE

Alteration of Plasma Protein Not a Cause.—The evidence obtained from exchange transfusion of blood between nephrotic rats and their normal litter mates invalidates the concept that proteinuria is due to alteration in the plasma proteins that makes them cross the glomerular membranes more readily than

normal proteins. If it were the alteration of the plasma proteins (dysproteinemia) that caused leakage of plasma proteins through the glomerular membranes and brought about the proteinuria, the administration of blood from nephrotic rats at the peak of proteinuria should have induced a similar proteinuria in the normal litter mates which received the blood which contained the supposedly altered proteins. Similarly, if the extracted urinary proteins were in the urine of nephrotic rats because alteration in their structure had facilitated their leakage through the glomerular membranes, these same proteins should have gone into the urine of the normal rats which received them. The experimental findings militate strongly against the concept that proteinuria is caused by alteration in plasma proteins.

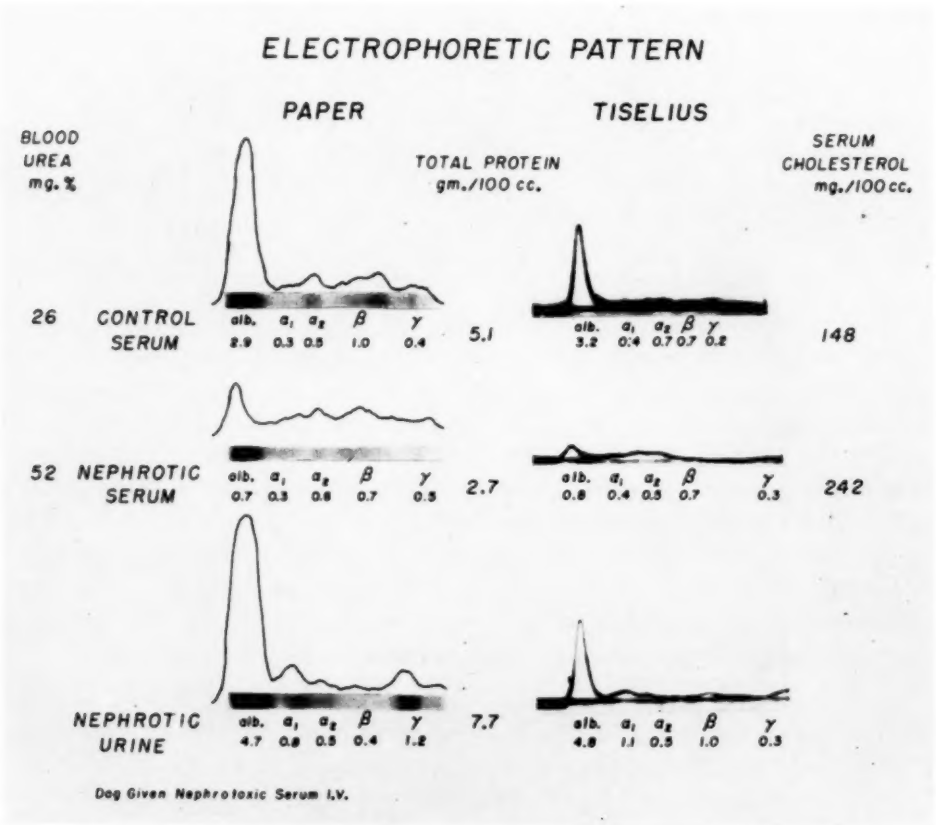
ELECTROPHORETIC PATTERN
PAPER
TISELIUS
BLOOD UREA mg. %
TOTAL PROTEIN gm./100 cc.
SERUM CHOLESTEROL mg./100 cc.
26
CONTROL SERUM
alb. 2.9 α1 0.3 α2 0.5 β 1.0 γ 0.4
5.1
alb. 3.2 α1 0.4 α2 0.7 β 0.7 γ 0.2
148
52
NEPHROTIC SERUM
alb. 0.7 α1 0.3 α2 0.6 β 0.7 γ 0.5
2.7
alb. 0.8 α1 0.4 α2 0.5 β 0.7 γ 0.3
242
NEPHROTIC URINE
alb. 4.7 α1 0.8 α2 0.5 β 0.4 γ 1.2
7.7
alb. 4.8 α1 1.1 α2 0.5 β 1.0 γ 0.3
Dog Given Nephrotoxic Serum I.V.

Fig. 1.—The electrophoretic patterns of serum and urinary proteins in a nephrotic dog as produced by paper chromatographic and Tiselius procedures. Note the similarity of the characteristic pattern of the proteins in the serum and urine of the nephrotic animal.

In their discussion of proteinuria, Bradley and Tyson[10] declared unconvincing the evidence that plasma contains abnormal proteins during this state. The finding that plasma albumin, separated by precipitation, has a higher molecular weight and lower osmotic pressure than is normal for albumin[11, 12] could be attributed to the presence of a larger proportion of contaminating globulin.

The same explanation can be given for the differences in fractions obtained by centrifugation.[13] The presence of immunologically distinct proteins was suggested by the inability of specific antiserums to plasma albumin and globulin to precipitate proteins from nephrotic subjects quantitatively.[14-16] It has been shown, however, that the unprecipitated portion of the albumin fraction is globulin. Alving and Mirsky[17] reported the presence of cystine-poor albumin in plasma from nephrotic subjects from which globulin had been removed by precipitation. However, Brand and co-workers[18] have shown that alpha globulin, the usual contaminant, contains very little cystine. That probably is what Alving and Mirsky were dealing with rather than an abnormal protein.

Hayman and Bender[19] injected 50 to 100 ml. of plasma from nephrotic patients with very severe proteinuria into 3 normal subjects. No proteinuria was produced in any of the normal subjects. Rusznyák and Németh[20] perfused the kidneys of dogs with Ringer's solution to which blood from nephrotic patients was added. No protein was excreted in the urine produced by these perfused kidneys. No immunochemical difference was noted between the albumin in plasma, ascitic fluid, or urine of nephrotic children.[21]

Protein Not Secreted by Tubular Epithelium.—For the hypothesis that proteinuria is produced by secretion of the proteins into the lumen of the tubules by the tubular epithelium, one of two conditions is prerequisite: either the presence of adequate amounts of protein in the interstitial fluid within the kidney, or the presence of disease which causes abnormal permeability of the peritubular capillaries to protein. Strong evidence against the hypothesis of protein secretion by the tubular epithelium is provided by the fact that proteinuria is minimal or absent in pyelonephritis, although peritubular capillaries are involved in the inflammatory process, and by the demonstration of protein coagula in Bowman's capsule.[9] Bieter's[5] inability to produce proteinuria in aglomerular fish militates against protein secretion by tubular epithelium.

Permeability of Glomeruli.—Therefore, of the various hypotheses presented for the mechanism of proteinuria there remains only the one based on damage to the glomerular capillary walls that increases their permeability and permits leakage of plasma proteins with the glomerular filtrate into the tubular lumen in amounts which exceed the maximal reabsorptive capacity of the tubular epithelium. Leakage of plasma proteins through abnormally permeable glomerular capillaries is the basic cause for appearance of protein in the glomerular filtrate.

Limited Tubular Reabsorption.—However, the epithelial cells of the renal tubules have an active part in the process. Overwhelming evidence has accumulated in recent years to support the importance of the role played by the epithelium of the renal tubules in the reabsorption of proteins from the glomerular filtrate. In an extensive review, Rather[22] presented convincing evidence that the cells which line the proximal convoluted tubules can reabsorb protein from the glomerular filtrate. The dye, T-1824, forms a stable blue complex with plasma proteins. The injection of T-1824 labels the plasma proteins to which

this dye is attached, absorbed, or combined. The dye, with the plasma proteins it tags, is found concentrated in the cells of the proximal convoluted tubules.[23, 24] This is evidence for tubular reabsorption of the plasma proteins from the glomerular filtrate. Sellers and co-workers[25] have demonstrated that urinary proteins have the same electrophoretic and solubility characteristics as the alpha and beta globulins of serum. If these large molecules are able to pass through the glomerular membranes, the smaller molecules of albumin certainly should. The plasma proteins constantly are passed in various quantities into the filtrate through the glomeruli and at the same time are continuously reabsorbed by the epithelial cells which line the renal tubules and are eventually returned to the bloodstream after some intracellular modification.

The glomerular filtrate in normal men amounts to about 180 L. per 24 hours. It contains, as variously reported, amounts ranging from 10 to 200 mg. of protein per 100 ml. of filtrate. Therefore, from 18 to 360 Gm. of plasma proteins pass through the glomeruli with the filtrate daily; and since practically no protein is normally present in the urine, the renal tubules must reabsorb that quantity of protein daily.

Oliver and co-workers[26] expressed the belief that whenever the capacity of the tubular cells to absorb proteins from the filtrate in the lumen of the tubules is exceeded by the amount or frustrated by the nature of the protein, an accessory mechanism of droplet formation appears. This mechanism is an intracellular process in which cytoplasmic elements, the mitochondria with their enzymes, and the absorbed protein unite to form droplets. Oliver and associates considered these droplets as foci of metabolic activity rather than cytologic structures of hyaline.[26] Owing to their original derivation from mitochondria, the droplets are highly active centers of protein metabolism which actively and specifically incorporate the proteins of the glomerular filtrate.

Recommended Hypothesis.—In the light of the evidence presented in the literature it can be stated that plasma proteins of varying amounts, depending on the status of the glomeruli, constantly pass through the glomeruli with the glomerular filtrate into the lumen of the tubules. The epithelial cells which line the tubules, on the other hand, constantly reabsorb these proteins. In a state of normaley the kidneys manipulate the filtered plasma proteins to such an extent that none is detectable in the urine by the ordinary clinical tests. It can be said, therefore, that for all practical purposes the urine normally contains no protein. However, in disease states when the permeability of the glomeruli is increased, plasma proteins leak through into the glomerular filtrate in amounts beyond the capacity of the tubular cell mass to reabsorb completely; consequently, a state of proteinuria ensues. Abnormal permeability of the glomeruli, exceeding the capacity of the tubular epithelium to reabsorb the filtered proteins, and disease of the epithelial cells of the renal tubules which reduces or annuls their ability to reabsorb proteins from the glomerular filtrate constitute the basic causes of the genuine proteinurias clinically encountered.

SUMMARY

The validity of the idea that a change in plasma proteins (dysproteinemia) causes proteinuria was assessed by producing experimental nephrosis in rats and then exchanging the blood between normal and nephrotic litter mates at the peak of nephrosis in the latter. Also, the proteins from the urine of the nephrotic rats were extracted and were given in Ringer's solution intravenously to normal rats. The patterns of urinary and plasma proteins were determined electrophoretically before and at frequent intervals after the exchange transfusion of blood or the intravenous administration of the extracted urinary proteins. Total proteins were quantitated chemically.

In spite of the exchange transfusion at the peak of proteinuria and hypoproteinemia, the nephrotic rats which received the normal blood from their litter mates continued to lose large amounts of plasma proteins into the urine, while the normal rats which received blood from their nephrotic litter mates did not show any evidence of increased loss of plasma protein into the urine. Furthermore, the normal rats which intravenously received the proteins extracted from the urine of their nephrotic litter mates did not show any increase in proteinuria.

The repeated findings that neither blood from nephrotic rats nor protein extracted from the urine of nephrotic rats caused any additional loss of protein into the urine of normal rats are considered convincing evidence against the hypothesis that a change in plasma proteins is the cause of proteinuria. Convincing evidence has accumulated in support of the hypothesis that proteinuria is due to damage of the glomerular membranes which leads to leakage of plasma proteins into the glomerular filtrate in amounts beyond the maximal reabsorptive capacity of the renal tubular epithelium. If the tubular epithelium is diseased, the leakage of the plasma proteins through the glomerular membranes need not be excessive to induce proteinuria.

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HEMAGGLUTINATION TEST FOR VIRAL HEPATITIS WITH SPECIAL REFERENCE TO NONSPECIFIC REACTIONS

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DURING the last two decades there have been numerous attempts to devise laboratory tests for viral hepatitis. In 1954, at the National Research Council Conference on Hepatitis, Havens[1] reviewed many of the reports on this subject. In this review are descriptions of the use of serum, feces, and tissue in procedures in which agglutination, precipitation, and complement fixation techniques are employed. More recently, he[2] has described an agglutination test in which fowl erythrocytes are employed. It was true then, as it is now, that there is no specific laboratory test for viral hepatitis.

In 1956, Morrison and Hoyt[3] described a serologic test in which red cells from the *Macaca rhesus* monkey are used. They had studied the hemagglutination reaction with cells from the mouse, sheep, steer, chicken, rabbit, guinea pig, horse, and various strains of monkeys. Of all the cells tested, those of the rhesus monkey appeared to be most promising. They reported that 86 per cent of their acute infectious hepatitis and serum hepatitis sera had a positive reaction when mixed with rhesus red cells. This was in sharp contrast to the control sera which reacted at a rate of 14 per cent. They concluded that the test showed promise.

Later in 1957, Rubin[4] described a modification of the Hoyt-Morrison test, and stated that there was some evidence that the hemagglutination was viral agglutination. If this were true it would mean that the test might be capable of great specificity. Rubin and co-workers believed that the agglutination might be viral because it was prevented by heating the serum, disappeared after the addition of beta-prone and was lessened by addition of convalescent serum to the system. The studies reported in this article were made in an effort to evaluate the specificity of this hemagglutination reaction in relation to viral hepatitis.

METHODS AND MATERIALS

The test employed was essentially that used by Rubin; 1 per cent suspension of rhesus cells was mixed with serum to be tested. The mixture was incubated at 4° C. for 1 hour,

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and reincubated at 37° C. for 15 minutes. The agglutination titer was read from the sedimentation pattern. In addition, 47 sera were tested by the Hoyt-Morrison technique for purposes of comparison. In this test the rhesus red cell suspensions were 2 per cent, incubation was at 37° C., and hemagglutination was read after centrifugation. The materials studied were frozen sera which had been obtained during acute and convalescent stages of infectious and serum hepatitis. There were several control groups investigated: medical students, laboratory workers, postpartum patients, newborns, patients with liver diseases, pediatric patients, and patients over 60 years of age.

RESULTS

In only one of 47 sera tested was there a significant difference between the titers obtained with Rubin and Hoyt-Morrison techniques (Hoyt-Morrison titer 64, and Rubin titer 16). In all samples tested, the difference was no greater than 1 tube (twofold dilution).

Seventy-eight per cent of the sera from patients with hepatitis tested exhibited titers of 16 or higher (Table I). In sharp contrast, only 12 per cent

TABLE I. HEMAGGLUTINATION TESTS IN VARIOUS GROUPS

GROUP	NO. TESTED	POSITIVE REACTION*	PER CENT POSITIVE
Patients with hepatitis	41	32	78
Patients with other liver diseases	16	2	12
Medical students	18	12	67
Laboratory workers	50	36	72
Postpartum patients	40	22	55
Newborns	10	0	0
Pediatric patients	23	15	65
Geriatric patients	49	6	12

*For this purpose, a positive reaction is defined as a titer of 16 or higher.

of patients with other liver diseases had a positive test. When compared with some of the other control groups, the differences between the titers of serum from patients with hepatitis and of the control sera are less marked: medical students, 67 per cent positive; laboratory workers, 72 per cent positive; postpartum patients, 55 per cent positive; and pediatric patients 65 per cent positive. The sera from newborns did not agglutinate the rhesus erythrocytes and the sera from geriatric patients provided few positive tests.

DISCUSSION

The initial studies looked most promising since the differences between the reactions of the sera from subjects with hepatitis and of those from subjects with other liver diseases were striking. It was difficult, however, to explain the high reaction rate of the laboratory workers. It was first thought that it might reflect exposure to the virus through daily contact with blood and serum, and that many of these workers had suffered unapparent attacks of viral hepatitis. Another possible explanation was that exposure to poliomyelitis vaccine prepared in rhesus kidney cells might have produced antibodies capable of agglutinating rhesus red cells. These hypotheses were rejected when the high rate of positive reactions was noted among postpartum patients and medical students, and preliminary inquiries failed to reveal a correlation between

poliomyelitis vaccination and a positive test. It then occurred to us that the differences among the groups might reflect age differences. As can be seen in Table I, the greatest difference was between the group with hepatitis and the patients with other liver diseases. Eighty-three per cent of the patients with hepatitis in this study in which the age is known were under 50 years of age. Only one patient with hepatitis was over 60 years of age. By contrast, patients with other liver diseases are from the older age groups; most patients in this group had cirrhosis, gallstones, or carcinoma affecting the biliary passages. We then attempted to test this hypothesis, that the differences in the control groups reflected age differences. For this purpose, agglutination titers of sera obtained from random pediatric patients at Children's Hospital of Michigan were compared with those of sera from random patients over 60 years of age. The results of these studies are set forth in Table I and reveal a distinct difference on the basis of age. From these investigations, it appears that the antibodies which agglutinate the rhesus erythrocytes are not present at birth, appear at an early age, persist during active adult life, and tend to decrease in titer during old age. Similarly, the data suggest that the differences between reactions of sera of patients with viral hepatitis and individuals with other liver disease are related to age differences, and do not depend on the virus which causes hepatitis. The rhesus red cell agglutination test appears to be not specific for viral hepatitis.

CONCLUSIONS

1. Agglutination of rhesus erythrocytes appears not to be viral agglutination.

2. Titers of various control groups differ greatly; these differences can be correlated with age differences.

3. The rhesus red cell agglutination test appears to be not specific for viral hepatitis.

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SERUM ARGINASE ACTIVITY IN SUBJECTS WITH HEPATOCELLULAR DAMAGE

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LIVER cell damage or necrosis causes a rise in serum enzymes such as transaminases[1, 3, 6] and aldolase.[2] However, these enzymes are so widely distributed that injury to organs, such as the heart,[4, 5] muscles,[1] or pancreas[5] may induce striking increase of their serum activity.

Arginase, first described by Kossel and Dakin[7] in 1904, is known to be present mainly in the liver[8, 9] and only in small quantities in other tissues, including the kidneys,[8, 9] breast,[9] and blood cells.[10] The fact that the liver is the main source of this enzyme has induced us to perform assays of serum arginase activity (SARG) in dogs with liver necrosis caused by carbon tetrachloride, and in patients with acute and chronic liver disease and miscellaneous conditions. In this way we hope to find an enzymatic test specific to liver injury.

In order to establish the sensitivity of serum arginase as an index of liver damage, simultaneous determinations of serum glutamic oxalacetic transaminase (SGOT) were done.

METHODS

Five mongrel dogs were fed carbon tetrachloride (1 ml. per kilogram) through a gastric tube. They were then assayed periodically for (SARG) and SGOT activity.

Blood samples were obtained from normal individuals, patients with viral hepatitis, cirrhosis, obstructive jaundice, and miscellaneous conditions, in unoxalated tubes. Sera were processed immediately or kept frozen for no more than 5 days, and hemolyzed blood was discarded. Diagnosis was made on the basis of the clinical history, liver profile tests,[16-21] and liver biopsy, when this procedure seemed necessary. Glutamic oxalacetic transaminase activity was determined by the method of Sall,[11] and expressed as Karmen units at 25° C. Arginase activity was assayed by the following technique based on the method of Kochakian,[12] modified by Reynolds[10]:

Reagents.—

1. L+ arginine monohydrochloride 0.047M solution in glass distilled water, pH adjusted to 9.9.
2. Diethylbarbiturate buffer, pH 9.9.
3. Cobaltous chloride 0.0015M solution in glass distilled water.

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4. Hydrocloric acid 0.3N. The pH of the buffer solution must be adjusted to a point at which the addition of 1.0 ml. of this buffer to the arginase digest will lower its pH to 7.0.)
5. Stock standard urea solution (4 μM per milliliter).
6. Urease solution (1.0 Gm. urease, 1.5 Gm. Permutit, and 15 Gm. sodium chloride, made up to a volume of 50 ml. with glass distilled water, mixed and shaken for 30 minutes, allowed to stand at room temperature for 2 hours, filtered, and adjusted to pH 7).
7. Acid cadmium sulphate solution (5.77 Gm. of $3CdSO_4$—$8H_2O$ and 28.2 ml. of H_2SO_4 1N, brought to a volume of 1 L. with distilled water).
8. Sodium hydroxide, 1.1N.
9. Nessler reagent.[15]
10. Ghatti gum.

For each determination an unknown sample, a blank, a standard, and a blank for the standard were run. The unknown sample consisted of a mixture of 2 ml. of L+ arginine, 1 ml. of barbiturate buffer, 0.25 ml. of cobaltous chloride, and 0.75 ml. of distilled water. The tube was placed in a water bath at 38° C., and, after 5 minutes 1 ml. of serum was added. The blank was similar to the unknown sample plus 1 ml. of 0.3N HCl; upon addition of the serum, the blank was placed in a boiling water bath for 5 minutes to arrest enzyme activity. Both blank and unknown sample tubes were shaken, rubber-stoppered, and incubated at 38° C. for 6 hours. At the end of the incubation period 1 ml. of 0.3N HCl was added to the unknown sample, and the tube was placed in boiling water for 5 minutes.

The tubes were cooled at room temperature before addition of 1 ml. of phosphate buffer, and were then thoroughly mixed. The formed precipitate may be discarded either by centrifugation at 2,600 r.p.m. for 10 minutes or by filtration. The resultant supernatant or filtrate can be stored in the refrigerator for 2 days before exposure to urease.

Three drops of urease were added. Five-tenths milliliter aliquots of each blank and unknown sample supernatants, after equilibration of the tubes at 38° C. for 5 minutes, were incubated at 38° C. in a water bath for 30 minutes. At the end of the incubation period, 8.5 ml. of acid cadmium sulphate solution was added; 1 minute later 1 ml. of 1.1N NaOH solution was added. The contents of the tubes were mixed, allowed to stand 10 minutes, and were centrifuged at 2,600 r.p.m. for 10 minutes. To 2.0 ml. of the supernatant, 0.2 ml. of ghatti gum and 5 ml. of Nessler reagent were added.

A standard containing 4 μM of urea in 1 ml. of water instead of serum, and a blank for this standard were carried through the same procedure. Five minutes after addition of the Nessler reagent, all the tubes were read after the colorimeter had been set at zero with the standard blank at 470 ml. The tubes were read against the standard blank. The amount of urea formed because of arginase activity was then calculated by subtraction of the value given by the unknown blank from the unknown sample.

Arginase activity is expressed in micromoles per milliliter of serum.

RESULTS

Animal Experiments.—The serum of normal dogs before the administration of carbon tetrachloride (CCl_4) showed no arginase activity. All 5 dogs had a significant increase of serum arginase activity after receiving CCl_4. The peak activity was observed between 48 and 72 hours, and the values ranged between 18 and 57 μM per milliliter of serum (Table I).

The SARG was transient, disappearing rapidly between 120 and 180 hours. SGOT rose from normal values (5 to 40 Karmen units at 25° C.) before administration of CCl_4 to 500 to 900 Karmen units at 25° C. All dogs survived the CCl_4 intoxication.

Human Experiments.—Figs. 1 and 2 summarize the results of SARG and SGOT activities obtained in the 102 cases studied.

1. The serum of 20 so-called normal individuals showed no SARG (Fig. 1). SGOT activity in this group ranged between 3 and 40 Karmen units at 25° C. (Fig. 2).

2. No SARG could be registered in subjects with 16 other miscellaneous conditions (Fig. 1 and Table II). In 17 patients with surgically proved uncomplicated obstructive jaundice, some of them with moderately SGOT activity, no SARG was detected (Figs. 1 and 2).

TABLE I. EFFECT OF CARBON TETRACHLORIDE ADMINISTRATION IN DOGS ON SARG AND SGOT ACTIVITY

DOG	WEIGHT (KG.)	DOSE (CCl_4 ml.)	TIME*	SERUM ARGINASE (μM/ML. SERUM)	SGOT (KARMEN UNITS AT 25° C.)
1	16	18.0	56	57	950
2	13	12.5	72	35	595
3	14	14.0	72	43	580
4	20	20.0	48	18	620
5	15	15.0	62	30	945

*Hours after administration of CCl_4.

TABLE II. SARG AND SGOT ACTIVITY IN SUBJECTS WITH MISCELLANEOUS CONDITIONS STUDIED

SUBJECT	SARG	SGOT (KARMEN UNITS AT 25° C.)	DIAGNOSIS
1	0	35	Peptic ulcer
2	0	60	Heart failure
3	0	300	Myocardial infarction
4	0	140	Myocardial infarction
5	0	130	Myocardial infarction
6	0	40	Acute rheumatic fever
7	0	100	Acquired hemolytic jaundice
8	0	60	Metastatic carcinoma
9	0	55	Metastatic carcinoma
10	0	250	Sepsis (*Clostridium welchii*)
11	0	32	Pleurisy
12	0	35	Sepsis (staphylococcus)
13	0	20	Amebiasis
14	0	60	Hypertension heart failure
15	0	150	Sepsis (*C. welchii*)
16	0	30	Pneumonia

3. In 23 patients with hepatitis studied in different stages of the disease a rise of SARG was found in 18; it fluctuated between 0.45 and 16 μM of urea per milliliter of serum. In these cases only the first determination of SARG has been considered (Fig. 2).

Table III correlates SARG and SGOT with total bilirubin and the duration of jaundice in the patients with viral hepatitis studied. As can be seen in this Table, a high serum arginase was usually found, corresponding with a high SGOT and a short period of jaundice. Subject 21 is an exception; but it must be considered that this patient with a long period of jaundice had no treatment and kept on working as a housewife for a period of 1 month before her admission to the hospital.

However, the highest value of serum arginase encountered, although corresponding to elevated SGOT, did not correlate exactly with the highest transaminase activities found.

The highest values of SGOT activity in our experience, ranging up to 1,200 Karmen units, were observed in the group with hepatitis (Fig. 2).

4. Cirrhosis of the liver caused a rise of serum arginase in fewer cases than viral hepatitis, and it was found elevated in 16 of 27 subjects studied (Fig. 1). SARG in these cases ranged between 0.45 and 7 μM of urea per milliliter of serum.

Only a slight to moderate increase of SGOT activity was observed by us in this group of cirrhotic patients, as is illustrated in Fig. 2.

TABLE III. SARG AND SGOT ACTIVITY IN SUBJECTS WITH HEPATITIS STUDIED

SUBJECT	TIME*	TOTAL BILIRUBIN (MG. %)	FLOCCULATION TESTS	SARG (μM UREA ML./ SERUM)	SGOT (KARMEN UNITS AT 25° C.)	DIAGNOSIS
1	9	15.6	+	4.40	380	Viral hepatitis
2	21	3.9	+	0	80	Viral hepatitis
3	16	7.9	+	0	90	Viral hepatitis
4	14	2.0	+	3.00	110	Viral hepatitis
5	7	7.9	+	16.00	400	Viral hepatitis
6	6	12.0	+	3.10	600	Viral hepatitis
7	10	20.0	+	6.50	1.200	Viral hepatitis† acute yellow atrophy
8	12	22.0	+	2.10	480	Viral hepatitis
9	16	1.8	+	0	70	Viral hepatitis
10	10	20.0	+	5.00	455	Viral hepatitis
11	15	12.5	+	2.60	600	Viral hepatitis
12	26	5.0	+	0	65	Viral hepatitis
13	10	7.2	+	2.40	700	Viral hepatitis
14	10	4.5	+	0.45	750	Viral hepatitis
15	9	14.0	+	2.80	860	Viral hepatitis
16	9	6.2	−	0	155	Chlorpromazine hepatitis
17	15	6.0	+	0	100	Viral hepatitis
18	9	9.5	+	2.20	710	Viral hepatitis
19	9	13.0	+	5.60	720	Viral hepatitis
20	6	25.0	+	1.35	435	Viral hepatitis
21	30	11.3	+	3.60	308	Viral hepatitis
22	18	4.1	+	1.80	137	Viral hepatitis

*Time, days of jaundice.
†Autopsy.

Table IV summarizes some of the clinical and biochemical data in the group of 27 cirrhotic patients studied. It is obvious from the observation of this Table that most of the patients were in an advanced stage of the disease. However, in this small group no correlation could be observed between severity of the clinical and biochemical findings on one hand and SARG on the other. No correlation, either, is noticeable between SARG and SGOT in this group.

DISCUSSION

The above results show that arginase is liberated into the blood stream in subjects with experimental hepatocellular damage and also in subjects with such diseases as viral hepatitis and cirrhosis of the liver, where liver cell injury is known to exist.

TABLE IV. CLINICAL AND BIOCHEMICAL DATA IN THE 27 PATIENTS WITH CIRRHOSIS STUDIED

SUBJECT	ASCITES	COMA	TOTAL BILIRUBIN (MG. %)	DIRECT BILIRUBIN (MG. %)	TOTAL CHOLESTEROL (MG. %)	C.C.F.*	T.T.†	PHOSPH.‡	PROTH.§ (%)	SARG. μM UREA/ML./ SERUM	SGOT (KARMEN UNITS AT 25° C.)	DIAGNOSIS
1	Absent	Absent	7.5	3.1	318	+ +	11.2	14	70	3	180	Cholostatic‖
2	Present	Absent	3.0	9.85	185	+ + +	14	14	65	0	39	Posthepatitis¶
3	Absent	Absent	2.6	0.9	159	+ + +	17	4	41	0	60	Hemochomatosis¶
4	Present	Absent	2.4	8.8	151	+ +	15	3	32	0	69	Alcoholic
5	Absent	Absent	0.6	0.3	105	+	7.5	4	50	0	100	Alcoholic**
6	Absent	Absent	2.4	0.6	224	(−)	2.9	3.4	55	0	9	Alcoholic**
7	Present	Absent	3	1	103	+ + +	7.7	3.4	45	5	28	Alcoholic
8	Present	Present	4.2	1.6	154	+ + +	12	5.7	28	3.1	257	Septal; unknown etiology¶
9	Present	Present	0.58	0.28	193	+ + +	8	2	30	0.9	49	Alcoholic
10	Absent	Absent	2.7	1.1	154	+ +	6.9	4.4	80	0	37	Alcoholic**
11	Present	Absent	5.1	1.8	134	+ + +	13.2	4.2	37	0.45	100	Alcoholic
12	Absent	Present	1.1	5.5	134	+ + +	13	6	47	7	207	Postnecrotic¶
13	Present	Present	1.8	8.2	134	+ + +	17	3.2	55	0	60	Alcoholic (florid)
14	Absent	Absent	3.4	1.7	182	+ +	61	5	32	7	88	Alcoholic
15	Present	Absent	8.7	4.3	111	+ + +	6.5	8	43	1.7	12	Alcoholic¶
16	Absent	Absent	0.5	0	162	+ + +	5	3	20	0	28	Alcoholic
17	Present	Absent	0.96	0.18	83	+ +	7.5	4	30	7	32	Alcoholic
18	Present	Present	1.6	0.53	205	+ + +	16	3	40	4	60	Alcoholic¶
19	Present	Absent	2.1	0.86	133	+ + +	9.9	5	40	3.1	132	Alcoholic
20	Absent	Absent	8.9	3.4	200	+ +	15.6	14	50	3.1	60	Cholostatic‖
21	Present	Absent	2.1	0.96	99	+ + +	6.9	6	40	1.3	45	Postnecrotic¶
22	Present	Absent	0.7	0.2	145		3	6	23	1.8	45	Alcoholic
23	Present	Present	12.2	3.9	390	+	7	1.6	18	0	68	Alcoholic (florid)¶
24	Absent	Present	17.5	7.9	322	(−)	2.9	10	80	4	34	Chronic cholangiolitis**; hepatitis
25	Present	Absent	4.2	1.6	145	+ +	7.4	18	55	0	68	Cholostatic
26	Present	Absent	1.9	0.6	175	+ + +	8	29		2.8	36	Unknown etiology
27	Absent	Absent	2.7	1.1	154	+ + +	6.9	4	35	0	37	Alcoholic

*Cephalin-cholesterol floculation test.
†Thymol turbidity test.
‡Alkaline phosphatase; Bodonsky units.
§Prothrombin concentration.
‖Surgical biopsy.
¶Autopsy.
**Needle biopsy.

The fact that as yet no serum arginase has been encountered in normal people and in subjects with illnesses other than liver disease is easily explained by the lack of this enzyme in significant amounts in other organs, and corroborates the possibility that this test may be quite specific for hepatocellular damage.

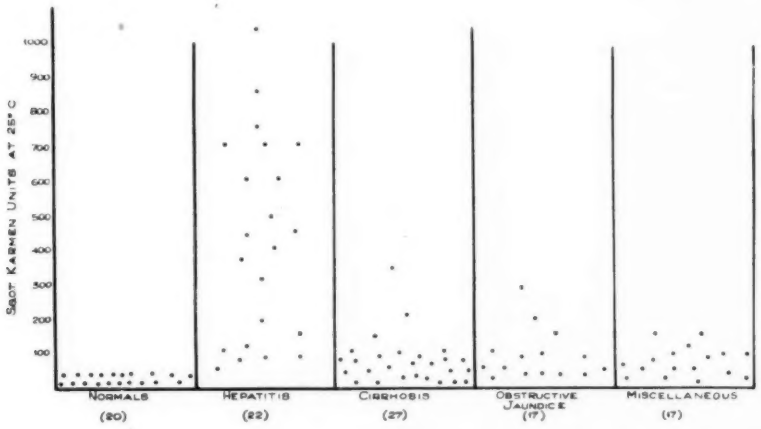

Fig. 1.—SARG levels in 102 cases studied. Each dot represents the highest level observed in a single case. The number under each bar refers to the total number of cases in the particular category cited.

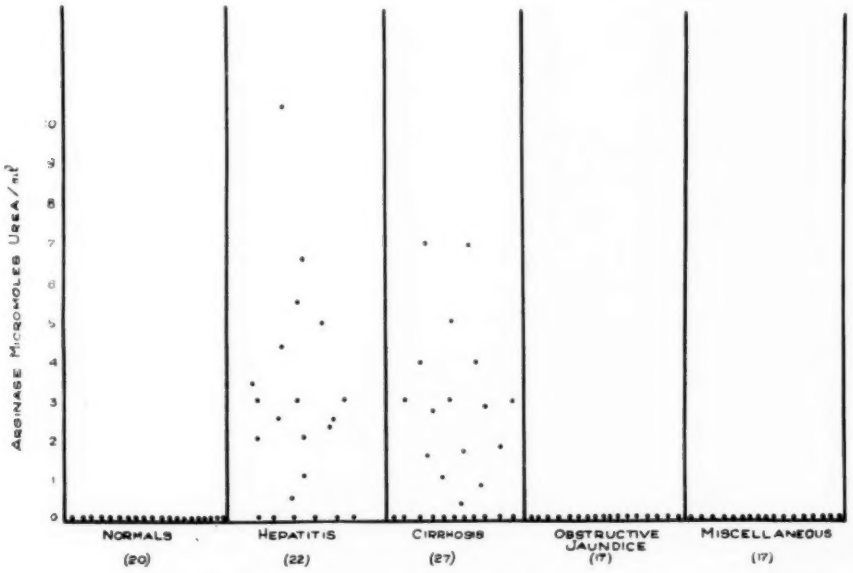

Fig. 2.—SGOT activity in 102 cases studied. Each dot represents the highest level observed in a single case. The number under each bar refers to the total number of cases in the particular category cited.

As was expected, more subjects with viral hepatitis than with cirrhosis showed elevated serum arginase, and the highest values were observed in subjects with hepatitis. In subjects with viral hepatitis and experimental hepatic

injury the increase of SARG was in most cases associated with a high SGOT value. This fact may suggest that the same mechanism is reponsible for the release of both enzymes, but our results in subjects with cirrhosis do not support this suggestion. In the group of patients with liver cirrhosis a high serum arginase was associated both with normal or moderately elevated SGOT; no clear-cut relationship could be drawn either with the clinical data or the rest of the biochemical data in these cases. Thus, in our present state of knowledge the mechanism responsible for an elevated serum arginase remains unknown.

In light of our experience, a SGOT of over 300 Karmen units and a positive serum arginase indicate acute liver damage in a patient with jaundice, particularly during the first 2 weeks of illness, whereas a low or moderately elevated SGOT with a positive or negative arginase activity does not differentiate acute from chronic liver damage.

Our observations regarding SGOT activity in these conditions agree with what has been previously reported in the literature by others.[5, 6] Our arginase results in cirrhosis also agree with those reported recently by Manning[14] on this disease, using another method.

SARG was absent in the patients with obstructive jaundice studied, although some of them had a moderately elevated SGOT.

These findings added to equally negative results reported previously by us[13] in dogs with ligation of the common bile duct, probably indicate that the finding of an elevated serum arginase should warrant caution in the diagnosis of uncomplicated obstructive jaundice.

SUMMARY

1. Serum arginase activity (SARG) was studied in dogs with experimental liver injury, in normal individuals, and in patients with hepatitis, cirrhosis of the liver, miscellaneous conditions, and obstructive jaundice.

2. Serum glutamic-oxalacetic transaminase (SGOT) was assayed in the same individuals, and was compared with the serum arginase activity.

3. In 5 dogs with moderate hepatocellular damage caused by carbon tetrachloride high values of serum arginase activity were found.

4. In the 20 normal individuals, the 16 individuals with miscellaneous conditions, and the 17 subjects with obstructive jaundice no arginase activity was found in the serum.

5. Sixteen patients with hepatitis of 22 cases studied showed elevated serum arginase activity.

6. Serum arginase activity was assayed in 27 subjects with cirrhosis, and was found elevated in 16.

7. Serum arginase activity roughly parallels SGOT activity in subjects with acute hepatocellular damage, but is absent from serum in other subjects with conditions which may have high SGOT activity.

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THE INFLUENCE OF HYPERTENSION ON SUSCEPTIBILITY OF THE RAT KIDNEY TO INFECTION

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SINCE the relationship between hypertension and pyelonephritis was clearly established by the studies of Wiess and Parker in 1939, many investigators have probed possible relationships which might exist between these often associated diseases. Clinical and animal studies recently reported upon have suggested that patients with pre-existing hypertension and no history or clinical evidence of renal disease harbor and, perhaps, incur bacterial infections of the kidney in significantly higher numbers than might be anticipated.[1-3] These reports prompted an animal experiment designed to test this hypothesis.

METHODS AND MATERIALS

Male Wistar rats, weighing approximately 125 grams, were made hypertensive by the method of Page.[4] The right or left kidney was exposed in alternate animals via a flank incision, and with purse-string suture a cellophane cap was applied. These animals comprise the "operated group." "Sham operated controls" were subjected to flank incision and exposure of either right or left kidney; without further manipulation the wound was closed. Light ether anesthesia was used during all operations and, postoperatively, 20,000 U. procaine penicillin was given intramuscularly. An equal number of animals were designated "unoperated controls." All animals were housed in pairs under identical conditions. All animals were fed a commercial dog pellet† which contained not less than 25 per cent crude protein, 7 per cent crude fat, 40 per cent nitrogen-free extract, and not more than 4 per cent crude fiber. Not more than 0.5 per cent iodized salt was present. Drinking water was 2 per cent saline. All animals were allowed to eat and drink ad libitum on this diet for the duration of the experiment.

For the purpose of this paper, hypertension is defined as an increase in systolic pressure of not less than 25 mm. Hg, as determined by occlusive tail plethysmography. All the animals were lightly anesthetized with ether during the blood pressure determinations; none were warmed prior to the measurement. The experiment extended for a 5 month period, June to October, 1958.

At the termination of the experiment, 39 control animals (24 not operated upon and 15 "sham operated controls") showed arterial pressures in ranges of 75 to 110 mm. Hg, with an average pressure of 90 mm. Hg and a mean per cent increase of 2 mm. Hg during the period of observation. The group of 39 animals which had been operated upon showed

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arterial pressures in ranges of 80 to 167 mm. Hg with a mean per cent increase of 32 mm. Hg. Twenty-four of the 39 animals operated upon (62 per cent) were hypertensive at the end of the experiment. Random arterial pressure recordings made throughout the experimental period showed that a larger percentage became hypertensive but failed to sustain the elevation of blood pressure. This phenomenon has been observed by others who used unilateral ischemia to induce experimental hypertension.

One milliliter of an inoculum of a virulent culture of *Escherichia coli* which contained 2×10^9 bacteria per milliliter was injected in the tail vein 5 days before the animals were lightly anesthetized (ether) and killed by hemorrhage. This interval was considered optimal to allow any histologic changes of acute pyelonephritis to develop.

Formalin-fixed kidneys were sectioned and stained with hematoxylin and eosin. Although gross infection was present in some cases, microscopic criteria were required for the diagnosis of pyelonephritis. These criteria include: (1) intraluminal and peritubular polymorphonuclear leukocytic infiltration, (2) frank abscess formation, (3) periglomerular leukocytosis, and (4) colloid casts. Perivascular collections of lymphocytes were not considered significant in this material.

All slides were independently reviewed by the authors and the Department of Pathology. There was agreement on all animals deemed infected. Fig. 1 is an example of the sort of pyelonephritis seen in the material.

RESULTS

The results are summarized in Table I. There remained at the end of the experiment 24 "unoperated controls," 15 "sham operated controls," and 39 animals operated upon (vide supra).

Of the 39 animals in the group operated upon, 24 (62 per cent) were hypertensive at the time bacteremia was induced. Eight (33 per cent) of the hypertensive animals subsequently had histologic evidence of pyelonephritis. There was no evidence of arteriolar hyperplasia or of nephrosclerosis in any of the material.

None of the 39 control animals was hypertensive and 5 (13 per cent) became infected.

TABLE I.

ANIMAL GROUP	ANIMALS (NO.)	NORMOTENSIVE	HYPERTENSIVE	ANIMALS INFECTED		
				TOTAL	NORMOTENSIVE	HYPERTENSIVE
"Unoperated controls"	24	24	0	2	2	0
"Sham operated controls"	15	15	0	3	3	0
"Operated group"	39	15	24	11	3	8
Totals	78	54	24	16	8	8

If one compares normotensive (39 controls and 15 nonhypertensive animals which had been operated upon) and hypertensive animals, it is evident that 8 of 24 (33 per cent) hypertensive and 8 of 54 (15 per cent) normotensive animals developed histologic evidence of pyelonephritis. Even though the incidence of pyelonephritis was more than twice as great in the hypertensive group, the difference is not statistically significant ($X^2 = 2.44$, $P = 0.13$). If the 15 nonhypertensive animals in the group operated upon are excluded, 5 of

39 (13 per cent) control and 8 of 24 (33 per cent) hypertensive animals are infected and the difference between the hypertensive and the control group is of borderline statistical significance ($X^2 = 2.78$, $P = 0.10$). Three of the 15 (20 per cent) operated upon but normotensive animals developed pyelonephritis. When compared with the controls (5 of 39, or 13 per cent) this suggests the possible conclusion that the history of operation does not affect the development of pyelonephritis in these animals, and the presence of elevated arterial blood pressure is the significant variable.

The kidneys of control animals at the time of autopsy weighed from 1.0 to 1.9 grams (mean value), whereas the intact kidney of the animals which had been operated upon weighed from 1.2 to 3.3 grams. There was no correlation between kidney weight and susceptibility to infection.

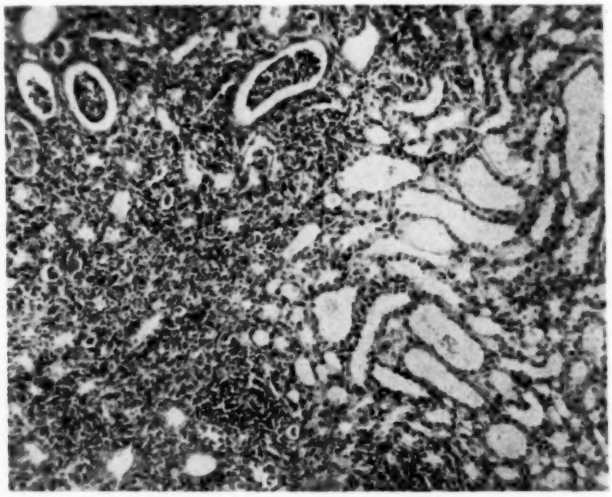

Fig. 1.—An intramedullary microabscess in the kidney of a hypertensive rat 5 days after the inoculation of *E. coli*. (×100; reduced ¼.)

DISCUSSION

The data presented suggest, but do not prove, that experimentally produced hypertension in rats renders these animals more susceptible to the development of pyelonephritis than a comparable group of normotensive animals when both are subjected to significant bacteremia. No changes of arteriolar nephrosclerosis were found microscopically in these kidneys. Those animals hypertensive for varying periods prior to injection of the bacteria, but not hypertensive at the time of bacteremia, developed pyelonephritis in essentially the same percentage of cases as did the control animals. The presence of hypertension is the significant variable. The absence of an apparent anatomic lesion allows one to speculate that changes in function rather than structure, at least at the level of light microscopy, play the major role in the increased susceptibility to infection of the rat kidney in experimental hypertension.

Huckabee[5] has presented data which compare rates of anaerobic metabolism of the kidneys in hypertensive and in normotensive patients. He concluded that the kidneys of the hypertensive group were abnormally dependent upon anaerobic metabolism because of relative inadequacy of oxygen supply to the urine-producing cells of the renal tubules. This opens an area for further study which may correlate with the susceptibility to infection of the kidney subjected to systemic arterial hypertension.

These findings are in agreement with those of Woods[6] and Shapiro.[7] Woods produced pyelonephritis in 23 of 33 hypertensive Sprague-Dawley rats (67 per cent) but in only 3 of 33 (9 per cent) normotensive controls with a DOCA-saline model. Diffuse arteriolar sclerosis preceded infection and was notable in the kidneys of these animals. He concluded that DOCA-saline-induced hypertension renders rats more susceptible to hematogenic renal infection.

Shapiro,[7] also using the DOCA-saline model, reported no gross pathologic lesions in kidneys of 15 normotensive rats infected with *E. coli* with positive renal cultures in 20 per cent. He further noted that 30 per cent of 17 hypertensive animals developed histologic pyelonephritis with positive cultures in 38 per cent.

After this study had been completed, Shapiro[8] published an abstract amplifying his initial study. He placed a clip on one renal artery, produced hypertension (126 ± 18 mm. Hg), and demonstrated acute pyelonephritis after inoculation of *E. coli* without renal massage in 47 per cent of hypertensive and 5 per cent of normotensive rats. These results offer further confirmation of the hypothesis that hypertension may precede and predispose to pyelonephritis in the experimental animal.

In an abstract, Grieble, Johnston, and Jackson[2] report that ambulatory patients with hypertension (followed for 2 to 20 months) had an attack rate for urinary tract infections of 4.5 per cent per year. This also suggests greater susceptibility of the hypertensive patient to infection. Smythe[9] has found that 13 of 77 previously unstudied hypertensive subjects had unsuspected evidence of pyelonephritis, whereas 8 of 77 carefully matched controls had infected urines.

It appears that clinical and experimental evidence is accumulating which suggests the relationship between pyelonephritis and hypertension may at least partially represent increased susceptibility of the hypertensive kidney to infection.

SUMMARY AND CONCLUSIONS

Hypertension was experimentally produced in rats by saline feeding and capping one kidney with cellophane. Susceptibility to pyelonephritis was tested by injection of 2×10^9 *Escherichia coli* into a tail vein. Of 24 hypertensive animals 8 developed evidence of pyelonephritis, but only 5 of 39 control animals became infected. These data suggest that the kidney of the hypertensive subject may be more susceptible to infection than that of the normotensive subject.

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This paper could not have been written without the helpful cooperation of Anna Chrisanthis. Drs. H. R. Pratt-Thomas and Mackenzie Moore were kind enough to supervise the preparation of the slides and help with their interpretation. The bacterial inoculum was prepared by Frank Cordle of the Department of Bacteriology.

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PERSISTENT PARASITISM IN EXPERIMENTAL BRUCELLOSIS: ATTEMPTS TO ELIMINATE BRUCELLAE WITH LONG-TERM TETRACYCLINE THERAPY

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ALTHOUGH the majority of patients with brucellosis recover without suffering a period of prolonged chronic illness the disease still poses a problem in therapy. The antibioties are effective in the treatment of brucellosis. The Expert Committee on Brucellosis of the World Health Organization in reviewing the results of antibiotic therapy concluded that the clinical course was shortened, complications were lessened, and fatalities were reduced.[1] However, there is still a residual of patients, who suffer relapses after what is considered to be adequate therapy, or whose symptoms persist because of active disease.

There undoubtedly remain many unknown host-parasite factors responsible for continued illness in brucellosis. One factor appears to be related to the intracellular localization of the Brucella organisms in the tissues of the hosts.[2] In these sites the bacteria are probably protected against both drugs and humoral antibiodies.[3, 4] Brucellosis, in many respects, is like tuberculosis in which there is the persistence of microorganisms in the host whose tissues have acquired exquisite hypersensitivity to Brucella products, and symptoms are related to this acquired hypersensitivity.[5]

Over a period of several years several experimental models have been employed in our laboratory in the evaluation of antibrucella drugs. The results of these studies, along with those of others, have been reviewed elsewhere.[6] It was found that a reliable and practical in vivo procedure for screening drugs was through the use of experimentally infected mice. Chronic infection can be readily established in this species and the results of treatment can be reasonably quantitated.

Most investigators in the management of human brucellosis administer antibioties for a maximum of 3 to 4 weeks. Perhaps this period of medication is inadequate for all patients, and treatment should be prolonged, as in tuberculosis. In order to evaluate short-term versus long-term antibiotic therapy, the present study was carried out in which a chronic Brucella infection was established in mice, and then the animals were treated for 4 to 6 months. Because chlortetracycline and tetracycline* produced favorable results in the therapy of human brucellosis, major emphasis was given to an evaluation of the latter antibiotic in the present study.

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MATERIALS AND METHODS

Since the studies extended over a period of several months, there were slight variations in the procedures. These will be noted in conjunction with each of the several experiments.

Materials.—Populations of male or female ABC white mice, 8 to 10 weeks of age, and weighing 18 to 20 grams were obtained from Dr. John Bittner of the Division of Cancer Biology of the University of Minnesota. The mice were housed in wooden or stainless steel cages in an air-conditioned room. Drinking water was freely available at all times. The animals were fed Purina Chow Checkers, except when an antibiotic was administered orally. Under these circumstances, the antibiotic under investigation was mixed with Purina Chow meal.

Brucellosis was established in mice with a smooth culture of *Brucella melitensis* 368, a strain that had originally been isolated from the blood of a human patient. Employing a tube dilution technique, this strain was found to be sensitive to the bacteriostatic action of tetracycline at a level of 0.4 μg per milliliter. Organisms were grown on slants of Albimi Brucella agar* for 48 hours at 37° C. A saline suspension was prepared and quantitated turbidimetrically. Dilution was carried out to yield an inoculum of 50,000 organisms per 0.1 ml., which was injected intraperitoneally.

TABLE I. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	24 WEEKS	28 WEEKS	32 WEEKS	36 WEEKS	40 WEEKS
78,200	34,360	280	0	0	0	0	0	0	0
84,500	37,542	560	0	0	0	0	0	0	0
104,840	39,890	130	0	0	0	0	0	0	0
109,390	48,180	430	40	0	0	0	10	0	0
119,560	79,538	460	80	0	0	0	10	0	0
56,630	123,442	570	90	0	0	0	20	130	0
73,080	123,442	690	120	0	10	0	40	760	10
80,160	98,627	660	140	30	30	170	300	8,820	30
104,990	136,168	4,810	11,400	320	70	800	17,010	34,020	70
138,280	183,708	5,170	47,780	37,170	6,360	83,160	23,310	0	

TABLE II. RECIPROCAL OF BRUCELLA AGGLUTININ TITER AT DIFFERENT PERIODS AFTER INFECTION IN UNTREATED INFECTED MICE

NO. OF WEEKS AFTER INFECTION	BRUCELLA TITER
8	320
12	640
16	640
32	160
40	80

Each test was determined on the pooled sera of 10 mice.

Methods.—Unless otherwise stated, treatment of infected mice was not started until 4 weeks after infection with *Br. melitensis*. This delay in treatment after initiating the infection simulated the course of illness in human brucellosis more closely than the commonly employed experimental procedure of beginning therapy simultaneously with the introduction of microorganisms into the selected host. At the end of 4 weeks the mice were divided into 2 large groups, a control infected group, and a group treated for varying periods of time with antibiotics. Groups of mice were killed after 4 weeks of infection, which was at the beginning of the period of therapy, and at monthly intervals thereafter.

The mice were killed with chloroform; blood was obtained by cardiac puncture for Brucella agglutinins; and the spleens were removed aseptically. The spleens were ground

*Obtained from Albimi Laboratories, Brooklyn, N. Y.

in tubes which contained sterile saline and sand, and aliquots were placed on the surface of Albimi Brucella agar plates. After 4 days of incubation at 37° C. the colonies were counted, and the total number of colonies contained in the spleen were calculated.

At the completion of each experiment autopsies were carried out in the remaining mice. Cultures of the livers, spleens, and kidneys were done, and histologic sections were prepared from the livers and kidneys and stained with hematoxylin and eosin.

Experiment 1: The purpose of this experiment was to study the natural course of brucellosis in untreated mice.

A group of 100 infected mice served as controls for the treated animals. Following an intraperitoneal inoculum of 50,000 Brucellae, groups of 10 adult mice were killed every 4 weeks for 40 weeks. The mice remained well throughout the experiment and gained weight. The numbers of Brucella colonies recovered from the spleens of these untreated mice are shown in Table I. Four weeks after infection there was evidence of multiplication of the Brucellae. Some diminution in the number of colonies occurred by the end of 8 weeks, but during the following 4 weeks a marked decrease had occurred. Three months after infection a gradual drop in the number of colonies had taken place, and at the end of 40 weeks, 6 of 9 spleens were sterile. The colony counts were of a low magnitude in the 3 spleens from which Brucellae were recovered. No Brucellae were obtained from the livers and kidneys of the

TABLE III. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	24 WEEKS	28 WEEKS	32 WEEKS	36 WEEKS	40 WEEKS
56,630	0	0	0	0	0	0	0	0	0
73,080	10	0	0	0	0	0	0	0	0
78,200	20	0	0	0	0	0	0	0	0
80,160	20	0	0	0	0	0	0	0	0
84,500	30	10	0	0	0	0	0	0	0
104,840	30	30	0	0	0	50	0	0	0
104,990	60	50	10	0	30	500	10	0	0
109,360	90	60	20	0	40	10,710	20	0	10
119,560	160	100	50	340	900	16,380	170	750	30
138,280	210	1,630	10,000	30,870	1,840	345,870	8,190	159,360	40

Treated with tetracycline 60 mg. per kilogram per day from 4 to 8 weeks after infection.

TABLE IV. RECIPROCAL OF BRUCELLA AGGLUTININ TITER AT DIFFERENT PERIODS AFTER INFECTION IN MICE TREATED FOR 4 WEEKS WITH TETRACYCLINE

NO. OF WEEKS AFTER INFECTION	BRUCELLA TITER
8	640
12	640
16	640
32	160
40	40

Each test was determined on the pooled sera of 10 mice.

mice sacrificed at the end of 40 weeks. Sections of the liver revealed the presence of occasional granulomatous lesions, about 1 per low power field. No lesions were seen in the kidney sections.

Sera of 10 mice were pooled at monthly intervals and tested for Brucella agglutinins. As noted in Table II the titer reached a maximum of 1:640 at the end of 3 months, remained elevated for another month, and then began to decline, so that 40 weeks after infection the titer was 1:40.

Experiment 2: Mice were infected at the same time as those in Experiment 1 and then 4 weeks after infection tetracycline was administered orally for 4 weeks.

Groups of 10 mice were killed at the same time intervals as in the first experiment and similar bacteriologic studies were conducted. Tetracycline hydrochloride was mixed with Purina Chow meal so that each mouse received 60 mg. per kilogram per day in 5 Gm. of food.

The antibiotic mixture was prepared in lots of 1,000 Gm. and the feeding jars in each cage refilled daily. Previous pilot studies had shown that a mouse consumed about 5 Gm. of feed daily.

The results of tetracycline therapy in this group are presented in Table III. It will be noted that 4 weeks after infection had been initiated, the number of Brucella colonies in a group of 10 untreated mice was essentially the same as found in the previous experiment

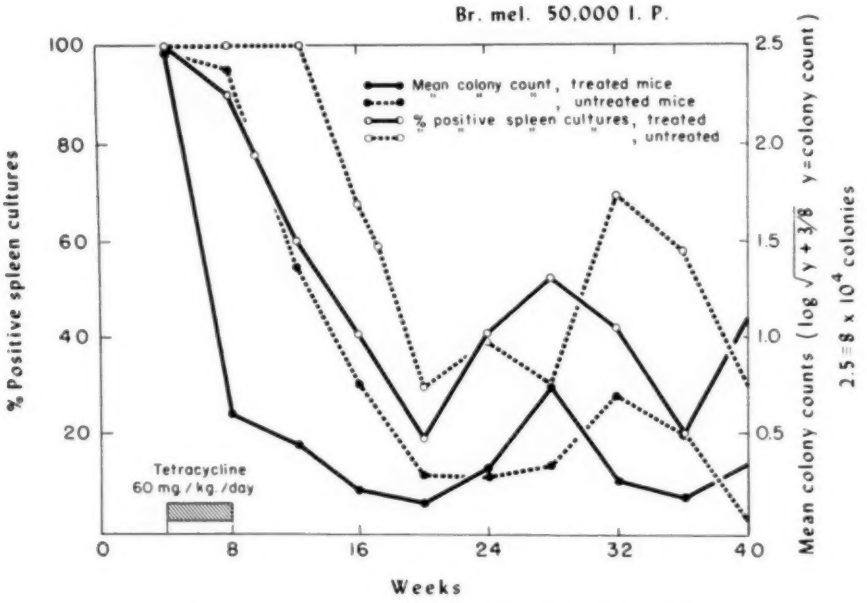


Fig. 1.—Tetracycline therapy in experimental brucellosis in mice.

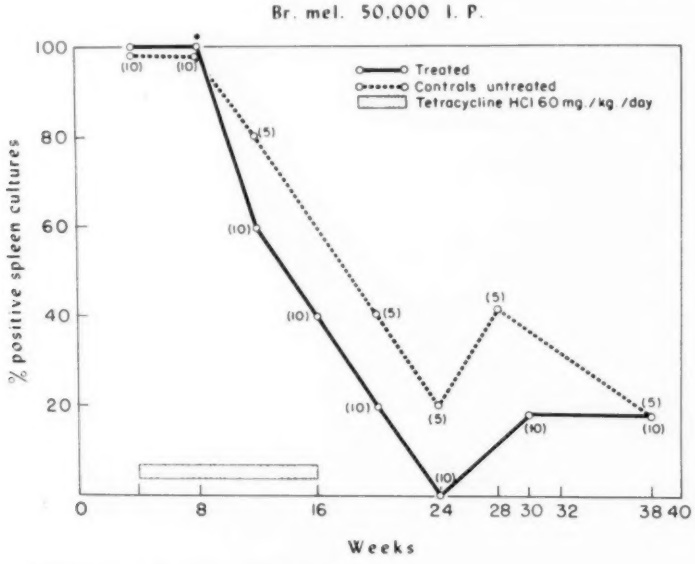
(Table I). The most outstanding result of treatment was the dramatic decline in the number of colonies of Brucella recovered from the spleens of 10 mice at the conclusion of 4 weeks of tetracycline therapy. Not only did therapy inhibit the multiplication of Brucellae, but eradication of the organisms from the spleen was also accelerated. The results of Experiments 1 and 2 are graphically shown in Fig. 1. The per cent of spleens from which Brucellae were cultured and the colony count per spleen from the untreated and treated animals are compared. Because of the incidence of high colony counts occurring in groups where 50 per cent or more of spleens were sterile, neither an average colony count nor a median figure would be indicative of the situation, so that the data were transformed using log $\sqrt{y + 3/8}$ to obtain a curve for the colony counts over a period of 40 weeks. Using a modified t test for possible unequal variances, the only significant difference between the treated and untreated groups was seen at 8 and 12 weeks, which were the periods at the end of therapy and 4 weeks later. After this there was no difference between the groups. There was no significant difference in the number of sterile spleens found in the 2 groups. These results emphasize the suppressive action of tetracycline on a Brucella infection.

The titers of Brucella agglutinins in the groups of treated mice showed essentially the same values for the untreated animals (Table IV).

At the completion of the experiment, which was 40 weeks after the mice had been infected, Brucella organisms were not cultured from the livers or kidneys of any of the treated mice. Histologic sections of the livers showed the presence of small compact granuloma, but they were less numerous than in the untreated controls. No kidney lesions were seen.

Experiment 3: The purpose of this experiment was to infect mice and then, after an elapse of 4 weeks, to treat them orally with tetracycline for 3 months.

The observations in this experiment were extended over a period of 38 weeks. One hundred and twenty-five female ABC mice which weighed 18 to 20 grams were infected with 50,000 organisms of strain *Br. melitensis* 368. At the end of 4 weeks the mice were divided into 2 groups. One group was fed with Purina Chow meal to which tetracycline hydrochloride had been added and mixed, so that each mouse ingested 60 mg. per kilogram of tetracycline daily. Groups of 10 treated mice were killed periodically for bacteriologic, serologic, and histologic studies. Groups of 5 untreated mice were studied periodically in a similar manner. The results of the Brucella colony counts of the spleens of the treated mice are shown in Table V. Two significant facts emerge from this study. First, as in the previous experiment (Table III), after 1 month of therapy there was a pronounced decrease in the number of Brucella colonies per mouse spleen as compared to the findings in untreated control animals (Table I). Second, after 24 weeks of observation, Brucella colonies were not isolated from the spleens of any of the 10 mice killed at this time. Apparently, a few colonies persisted in the tissues of the surviving mice since Brucellae were cultured from the spleens of 2 out of 10 mice on each of 2 subsequent periods. The important feature of this experiment is that tetracycline suppressed the multiplication of Brucellae to such an extent that if the experiment had been terminated at 24 weeks, or 4 weeks after the completion of treatment, it could have been concluded that 3 months of therapy completely eradicated



*Refers to colony counts in table.

Fig. 2.—Experimental brucellosis: 3 months' therapy.

Brucellae from the spleen. Comparative bacteriologic studies on the spleens of untreated and treated mice are shown graphically in Fig. 2. At the conclusion of the experiment, which was 38 weeks after the mice had been infected, cultures of the livers, kidneys, and lungs from 10 treated mice remained sterile.

Histologic studies of the livers in the treated mice showed infrequent and very small granulomas. The Brucella agglutinin titers were the same in the treated and untreated groups.

Experiment 4: The purpose of this experiment was to infect mice, and then, after 4 weeks, to treat them orally with tetracycline for 6 months.

One hundred female colored ABC mice were infected intraperitoneally with 50,000 organisms of strain *Br. melitensis* 368. Four weeks later the mice were fed 60 mg. per kilogram daily of tetracycline hydrochloride, which had been mixed with Purina Chow meal. Eighty mice were infected at the same time and used as an untreated control group for this and the following experiment. As in the previous experiments groups of mice were killed monthly, and bacteriologic, serologic, and histologic studies were carried out. Although the same daily dose of tetracycline was employed in this experiment as in the previous experiments the initial response to therapy was much better than in the previous studies (Table VI). After 2 months of therapy 80 per cent of the spleens were sterile. After the fifth and

TABLE V. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

MOUSE NO.	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	24 WEEKS	30 WEEKS	38 WEEKS
1	11,400	120	0	0	0	0	0	0
2	17,800	260	0	0	0	0	0	0
3	18,900	740	0	0	0	0	0	0
4	18,900	760	0	0	0	0	0	0
5	33,800	840	120	0	0	0	0	0
6	43,900	1,100	180	0	0	0	0	0
7	43,900	1,500	190	130	0	0	0	0
8	48,180	1,500	260	240	0	0	0	0
9	56,630	1,660	1,040	950	79	0	10	780
10	56,630	1,780	3,580	2,420	90	0	60	5,460

Treated with tetracycline 60 mg. per kilogram per day for 3 months from 4 to 16 weeks after infection.

TABLE VI. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

MOUSE NO.	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	24 WEEKS	28 WEEKS	32 WEEKS	36 WEEKS	40 WEEKS
1	4,500	110	0	0	0	0	0	0	0	0
2	5,600	120	0	0	0	0	0	0	0	0
3	24,800	140	0	0	0	0	0	0	0	0
4	26,700	190	0	0	0	0	0	1,040	0	0
5	29,900	720	0	0	0	0	0	1,500	0	0
6	31,800	720	0	0	0	0	0	4,380	40	
7	40,700	840	0	0	0	0	0			
8	43,600	950	0	0	0	0	0			
9	75,700	2,650	60	30	0	0	0			
10	75,700	3,900	80	70	Contam.	0	0			

Treated with tetracycline 60 mg. per kilogram per day for 6 months from 4 to 28 weeks after infection.

sixth months of therapy 100 per cent of the spleens were sterile. However, when mice were killed at the conclusion of therapy, Brucellae could still be cultured from some of the spleens. This experiment emphasizes again that tetracycline acts as an effective suppressive antibrucella agent. This is particularly obvious if the results with untreated mice (Table VII) are compared with those of the treated mice (Table VI). Cultures of the livers, kidneys, and lungs of the treated mice remained sterile at the conclusion of the experiment.

Histologic studies of the livers from mice killed 40 weeks after the mice had been infected, and after 6 months of treatment with tetracycline, showed an occasional very small and compact granuloma. Brucella agglutinins appeared in the treated animals to the same extent as in untreated controls.

Experiment 5: The purpose of the experiment was to treat the infected animals *intermittently* with 2 courses of tetracycline HCl administered orally in Purina Chow meal in a daily dose of 60 mg. per kilogram for 2 periods each of 4 weeks.

Female colored ABC mice were used. They were infected intraperitoneally with 50,000 organisms from the strain *Br. melitensis* 368. As in the previous experiments, mice were infected and a period of 4 weeks elapsed before therapy was instituted. The first course of therapy extended over 1 month, between the fourth and eighth weeks. At the end of the twentieth week, 40 mice previously treated for 1 month, were divided into groups of 5. One-half received a second course of oral tetracycline between the twentieth and twenty-fourth week. Thus, it was possible to kill at monthly intervals, groups of 5 mice that had been treated for 1 month only, and groups that had received an additional 1 month of therapy.

Appropriate untreated control mice were studied bacteriologically, serologically, and histologically. The results of the Brucella colony counts from the spleens are shown in Table VII. It is of interest to compare the results of this experiment with those of a similar experiment (Table I) in which untreated mice were also studied over a period of 40 weeks. In Experiment 1, Table I, male albino ABC mice were used, whereas in this experiment the controls consisted of female colored ABC mice. This distinction in the 2 populations might explain some of the minor differences in the results. The results were essentially the same in that there was a progressive elimination of Brucellae from the spleens over a period of several weeks, but Brucellae were not eradicated from all of the mice. Cultures of the livers, kidneys, and lungs remained sterile at the end of 40 weeks. Brucella agglutinins were present in the pooled sera of the mice all through the period of 40 weeks of observation. Histologic studies of the livers from the mice killed at the end of 40 weeks revealed more numerous and larger granulomas than noted in Experiment 1. This difference may reside in the 2 different genetic populations of mice that were used. No lesions were seen in sections of the kidneys.

TABLE VII. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.): UNTREATED CONTROL MICE

4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	24 WEEKS	28 WEEKS	32 WEEKS	36 WEEKS	40 WEEKS
17,800	1,480	0	0	0	0	0	0	0	0
18,900	6,360	60	0	0	0	0	0	0	0
19,900	7,630	90	50	0	0	0	0	0	0
39,890	8,820	150	80	0	0	0	0	0	0
33,800	11,400	450	90	0	0	0	10	0	0
37,170	22,900	460	540	40	10	10	10	0	0
43,900	23,310	590	570	750	50	20	40	120	50
47,780	34,020	840	980	1,400	70	30	110	130	3,120

The results obtained in the treated mice are shown in Table VIII. When this population of mice was treated orally for 1 month with tetracycline there was a progressive dimunition in the number of Brucella colonies that were cultured from the spleens. The results were less pronounced than those obtained in Experiment 2 (Table III). When groups of mice were treated orally for an additional 1 month with tetracycline, between the twentieth and twenty-fourth weeks after infection, additional suppression and elimination of Brucellae from the spleens were observed. In 2 groups of mice, after 2 courses of therapy, Brucellae could not be obtained from the spleens. But this intermittent therapy did not completely eliminate the Brucellae, since 2 of 20 spleens still yielded organisms. A comparison of the number of positive spleen cultures is shown graphically in Fig. 3. Data obtained from this experiment and from a previous experiment (Experiment 1, Table III) show that 2 intermittent courses of tetracycline of 1 month each are more successful in the suppression and elimination of Brucellae than a single course of 1 month's treatment. Cultures of the livers, kidneys, and lungs 40 weeks after infection remained sterile in both groups of treated mice. Histologic sections of the livers showed an occasional small granuloma in both groups.

TABLE VIII. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

MOUSE NO.	MICE TREATED 4-8 WEEKS					CONTROL TREATED 4-8 WEEKS (COL. 1) MICE TREATED 4-8 WEEKS AND 20-24 WEEKS (COL. 2)							
						24 WEEKS		28 WEEKS		32 WEEKS		40 WEEKS	
	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS	1	2	1	2	1	2	1	2
1	10,400	0	70	0	0	0	0	0	0	0	0	130	0
2	17,500	0	80	0	0	0	0	0	0	0	0	170	0
3	18,900	0	330	10	10	0	0	0	0	0	0	570	0
4	28,900	0	420	10	20	170	0	0	0	140	0	910	0
5	35,800	70	390	20	30	5,720	0	12	390	410	0	64,600	140
6	48,180	80	560	30	30								
7	48,900	280	700	100	50								
8	58,700	1,030	940	120	70								
9	59,630	1,880	5,040	360	530								
10	70,630	11,400	171,800	420	720								

Treated intermittently with tetracycline 60 mg. per kilogram per day from 4 to 8 weeks and from 20 to 24 weeks after infection.

Experiment 6: In all of the preceding experiments, therapy with tetracycline was not started until 4 weeks *after* the mice had been infected with Brucellae. Under these circumstances, even with prolonged tetracycline therapy the organisms could not be eliminated from the spleens of all of the mice. The present experiment was designed to determine whether the Brucellae could be eradicated more effectively if therapy was started before the onset of infection.

Sixty female ABC mice were inoculated intraperitoneally with 50,000 organisms of *Br. melitensis* 368. One half of the mice served as infected controls, and the other one half were fed 60 mg. per kilogram of tetracycline daily in Purina Chow meal beginning 1 day before infection and treatment was continued for 4 additional weeks. After 4, 8, and 12 weeks of infection, 10 mice from each group were killed. The titers of Brucella agglutinins were determined on hearts' blood at the end of each period; cultures of the spleens were made; and sections of the livers were examined microscopically.

Although the mice had received tetracycline at the time they were infected with a relatively small inoculum of Brucellae, the organisms succeeded in localizing and multiplying within the spleens (Table IX). However, tetracycline did have some suppressive effect since the number of Brucellae isolated from the spleens of the treated mice was definitely less than those cultured from the untreated group. The effect of therapy was particularly apparent 4 weeks after the conclusion of therapy, or 8 weeks after the infection had taken place. Although the experiment was terminated at the end of 12 weeks, Brucellae could still be isolated from the spleens in 4 out of 10 treated mice.

TABLE IX. COLONY COUNTS PER MOUSE SPLEEN AT INTERVALS AFTER INFECTION WITH BR. MELITENSIS (50,000 ORGANISMS I.P.)

MOUSE NO.	4 WEEKS	8 WEEKS	12 WEEKS
1	130	0	0
2	140	0	0
3	160	0	0
4	450	0	0
5	550	10	0
6	770	10	0
7	860	30	60
8	1,400	30	500
9	Contaminated	—	3,800
10	—	—	4,600
Untreated Control Mice.—			
1	10,000	730	0
2	51,000	3,600	0
3	74,000	6,900	30
4	80,000	40,000	1,900
5	97,000	58,000	2,500
6	110,000	86,000	3,800
7	160,000	89,000	5,700
8	130,000	120,000	—
9	150,000	130,000	—
10	200,000	170,000	—

Treated with tetracycline 60 mg. per kilogram per day for 4 weeks commencing 1 day before infection.

Immediate therapy did not interfere with the appearance of Brucella agglutinins. Sections of the livers stained with hematoxylin and eosin were examined for granuloma. Control and treated mice killed immediately after the conclusion of treatment showed marked differences. The livers of 9 of 10 untreated mice revealed granulomas; numerous large lesions were present in each section of 4 of the 9. Only 6 of 9 livers of the treated animals had granulomas. These were very small, and only 1 lesion was present in each section examined. Likewise, at the end of the experiment there were fewer hepatic granuloma in the treated group.

DISCUSSION

The course of brucellosis in experimentally infected mice has revealed that there is multiplication of the organisms for several weeks after the infection has been initiated; localization of the bacteria occurs particularly in the spleen. Over a period of several months the infected mice appeared healthy and gained in weight. The development of an efficient defense mechanism was reflected by the gradual suppression and elimination of the Brucellae from the spleens. But persistent parasitization of some animals did take place and Brucellae could be recovered up to 40 weeks after infection.

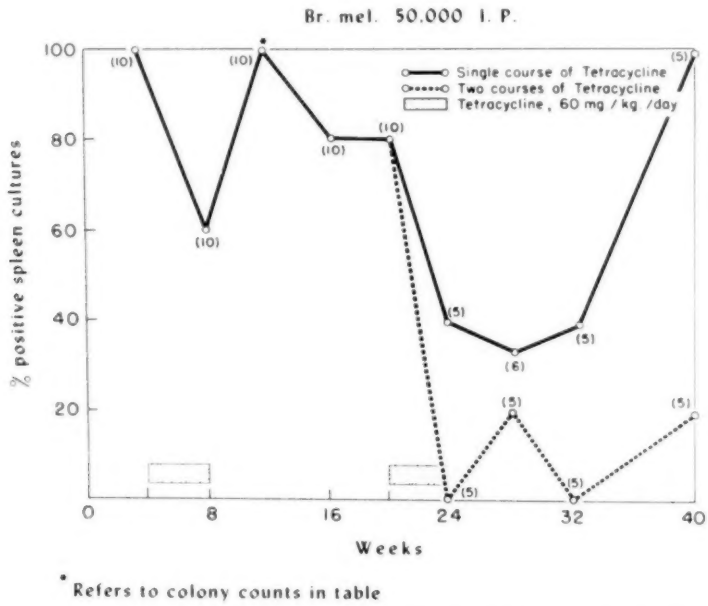


Fig. 3.—Intermittent therapy in experimental brucellosis in mice.

Chlortetracycline and tetracycline are bacteriostatic agents. Suppression of the growth of Brucellae by these antibiotics can be readily demonstrated in vitro. The present investigations have shown that tetracycline suppressed the multiplication of Brucellae in the tissues of mice having established infections, thus aiding the defense mechanism of the host in eliminating Brucellae from the tissues. This complementary effect of tetracycline was especially apparent in those mice which had received tetracycline continuously for 3 to 6 months, or which had been treated intermittently on 2 occasions for 4 weeks each time.

The similarity of brucellosis to tuberculosis is demonstrated by a comparison of the results in this communication with those of McCune and Tompsett.[7] These investigators reported that tubercle bacilli persisted in the spleens of mice after intensive and prolonged therapy with antituberculous agents. This persistence of the infection was not due to the appearance of drug-resistant

variants of the tubercle bacillus. In several different studies we have never demonstrated that the persistence of Brucellae in the tissues of treated animals was due to resistant organisms. We have encountered the appearance of resistant Brucellae in a treated human patient only on 1 occasion, which occurred after therapy with streptomycin.[9] Both streptomycin-resistant and streptomycin-dependent colonies of Brucellae were isolated from the blood stream of this individual. McCune, Tompsett, and McDermott[5] extended their observations on the therapy of tuberculosis in mice by the use of a combination of drugs. No tubercle bacilli were recovered from the tissues shortly after therapy had been completed. But when the tissues of treated mice were examined 90 days after the conclusion of the combined treatment, tubercle bacilli were isolated. This indicated that a latent infection had been established by the treatment. Similar observations were made in the present study with Brucella infected mice which were treated for a period of many weeks with tetracycline alone. At one period after treatment the spleens of all the treated mice remained sterile, but Brucellae were recovered from some mice killed at a later time after treatment. The experiments were not designed to test the effect of 2 drugs on the course of brucellosis. During the course of the study 1 large ancillary experiment was carried out in which mice were infected with *Br. melitensis* and treated with a combination of streptomycin and tetracycline for 1 month. Immediately at the conclusion of treatment, and for several weeks thereafter, Brucellae were not eradicated from the spleens of all of the mice. The results were quite similar to those obtained when mice were treated only with tetracycline for the same length of time.

Brucellae can persist in the tissues of apparently healthy animals, other than mice, for long periods of time. Naturally infected goats and cattle have been known to shed Brucellae in milk over a period of several years, although the animals have appeared to be in good health and there has been no decrease in milk production because of localization of the Brucellae in the udders. On rare occasions, Brucellae have been cultured from isolated lesions of human beings, who have not complained of ill health. But the persistence of pathogenic microorganisms in the tissues of an invaded healthy host is not unique for either brucellosis or tuberculosis. Many other species of bacteria, as well as rickettsiae, viruses, and fungi, can localize and remain for many years within the human host without manifestations of disease. The problem of the persistence of Brucellae in human tissues is that it is sometimes associated with relapses of the disease and a prolonged state of ill health.

SUMMARY

1. Populations of ABC mice infected with the tetracycline-sensitive strain of *Brucella melitensis* 368 were used as an experimental model for quantitating the suppressive action of the antibiotic under different dose schedules. A persistent infection was readily established by infecting the mice intraperitoneally with 50,000 to 100,000 organisms. The brucellae localized and multiplied in

the hosts, particularly in the spleens. The antibrucella effect of tetracycline was determined by starting therapy 4 weeks after initiation of the infection, and then determining Brucella colony counts on spleens removed from groups of mice after varying periods of therapy. In addition, titers of Brucella agglutinins were determined on the pooled sera of killed mice, and histologic studies were made on sections of the livers.

2. Colony counts on the spleens of untreated infected mice revealed that organisms mutiplied in the spleens up to 8 weeks after infection. Between 12 and 16 weeks after infection there was an abrupt decrease in the number of Brucellae, and after 16 weeks no Brucellae were recovered from the spleens of approximately 30 per cent of the mice. Although the numbers of Brucellae were small, organisms could still be cultured from about one third of the spleens 40 weeks after infection. A maximum titer of Brucella agglutinins was detected by the twelfth week after infection, and agglutinins could be demonstrated in lower titers after 40 weeks. Occasional granulomas were seen in the livers.

3. Four weeks after being infected groups of mice were treated orally with 60 mg. per kilogram of tetracycline HCl daily for 1 month, for 3 months, for 6 months, and in 1 experiment, intermittently, in which the mice received tetracycline for 1 month, and again for 1 month after an interval of 3 months. All of the experiments were terminated 38 to 40 weeks after infection. When treated for 1 month the outstanding feature was an enhancement in the suppression of the multiplication of Brucellae in the spleens. But at the end of the 40 weeks Brucellae were recovered from essentially the same number of spleens as in the untreated group. When the mice were treated for 3 to 6 months, suppression of the growth of Brucellae was noted as before and, in addition, Brucellae could not be recovered from the majority of spleens at the conclusion of the experiment. Intermittent therapy of 2 periods of 1 month each resulted in about the same degree of suppression as that obtained with prolonged continuous therapy.

Prolonged therapy did not alter the pattern of titers of Brucella agglutinins as compared to the untreated controls. Smaller and fewer granulomatous lesions were apparent in the livers of all of the treated mice, particularly in those mice that had received treatment over a long period of time.

4. In 1 experiment tetracycline therapy was started the day before the mice were infected and treatment was continued for 4 weeks. Suppression of the infection was again observed but 12 weeks after infection Brucellae could be cultured from 40 per cent of the spleens. The number of liver lesions was markedly diminished in the treated group.

5. Experimental brucellosis in mice reflects the persistent parasitization that can occur in apparently healthy animals. The present investigations also emphasize that latent infection can persist over a long period of time after intensive suppressive therapy. But these features of the host-parasite relationship are not unique for brucellosis.

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ON THE SURVIVAL TIME OF LEUKOCYTES IN LEUKEMIC PATIENTS

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ATTEMPTS have been made for a long time to study the survival time of leukocytes in circulation by the use of a wide variety of techniques.[1-4] Many of these experiments give extremely short life spans, varying from a fraction of an hour to a fraction of a day. The probability of disappearance of white cells to extravascular spaces and capillaries and of manipulative damage in transfusion experiments was ignored.

More recently radioactive tracers have been used to tag the desoxyribose nucleic acid (DNA) content of leukocytes and to measure the prevascular and intravascular life span of leukocytes from DNA turnover data, obtained either with chemical procedures or autoradiographic techniques. Life spans of normal leukocytes,[5-9] and of those in various diseased states such as leukemias and leukemoid reactions[10-14] have been deduced in this way. Whether a definite life span of white cells can be correctly obtained from these techniques has been the subject of much discussion.[15, 16] Possibly such measurements give only an indication of the duration of the prevascular and vascular phases.[15]

This paper contains an account of the study of the growth in activities in different fractions of blood and white cells after administration of a tracer dose of radioactive phosphorus. The study is concerned with 3 cases of chronic myeloid leukemia and 1 case of chronic lymphatic leukemia. An attempt has been made to derive the average period of stay of leukemic leukocytes in marrow and in circulation.

MATERIALS AND METHODS

Four untreated patients with chronic myeloid leukemia and 1 patient with chronic lymphatic leukemia were given orally, in a fasting condition, a tracer-dose of P^{32} in the form of isotonic Na_2HPO_4. No food was allowed until 4 hours after the tracer-dose was administered and blood samples were drawn at short intervals until 12 hours after the dose, and then daily every morning with the patient in a fasting condition. Blood counts including full hemogram were done daily.

The collected blood was divided into 3 parts and the following measurements were made: (a) activity per milliliter of whole blood, (b) activity per milliliter of plasma after separation of cells by centrifugation, and (c) activity per milliliter of packed white and red cells and in different fractions of white cells.

The white cells were separated by adding dextran (6 per cent in N saline 1:1 v/v) and allowing the whole mixture to stand for an hour for differential sedimentation

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of cells. The supernatant, rich in white cells, was pipetted off, and was spun at 1,000 r.p.m. for 15 minutes. The supernatant containing platelets was discarded, and the sediment was resuspended in normal saline and centrifuged at 3,000 r.p.m. for 15 minutes. The process was repeated twice. The final white cell sediment was suspended in normal saline in a graduated tube and diluted to a specific volume. The number of white cells per milliliter was counted.* Repeated daily counting showed that 1 ml. of packed white cells contained an average of 1.4×10^9 cells in the cases of myeloid leukemia, and 2.3×10^9 cells in the case of lymphatic leukemia.

Desoxyribose nucleic acid was extracted quantitatively from a measured volume of white cells by the method of Schmidt and Thannhauser[17] with the modification of Schneider.[18] All the experimental procedures for DNA extraction were carried out at 0° C., except the intermediate step of separation of lipids, which was carried out at room temperature. The separated DNA and ribosenucleic acid (RNA) in the final step were tested chemically (orcinol reaction for pentose, and diphenylamine reaction for desoxypentose) to show that the DNA fraction contained no RNA and that the filtrate which contained RNA was free from DNA. The filtrates in the different stages of the DNA separation were collected to determine the activities in acid soluble phosphorus, phospholipid, and RNA phosphorus. The precipitated DNA was dissolved in a known volume of N KOH and the activity of DNA in 1 ml. packed WBC was determined.†

All the activity measurements were done with a Tracerlab TGC-1 end window G.M. counter with a background of about 23 c.p.m.; the counts were always more than 2,000. The counts obtained were converted to microcuries by comparison with the counts from 1 ml. of a standard P^{32} solution of known strength. All counts were corrected to 0-day.

RESULTS

In all the cases studied here, the plasma activity per milliliter reached the peak within 3 to 6 hours after application of P^{32}. The total activity in the plasma at the peak was 6 to 7 per cent of the total administered dose. This plasma peak was normalized to 100 in each case; the average variation of the activity in plasma for the 3 myeloid subjects has been shown in Fig. 1. The plasma activity diminished very rapidly within the first few hours, as shown in Fig. 2. After 7 days the decrease in plasma activity seemed to follow an exponential law with a rate constant of 0.024 day^{-1}.

Fig. 1 also shows the activity variations with time, of whole blood per milliliter, of plasma per milliliter, of WBC per milliliter packed cells, of RBC per milliliter packed cells, and of DNA per milliliter packed WBC in patients with chronic myeloid leukemia. These are the average values for the 3 patients with chronic myeloid leukemia. Activities in these fractions of blood have been expressed as per cent of the peak activity in plasma. The specific activity of whole blood reached its peak value (40 per cent of plasma peak) in 24 hours, and that in RBC (about 100 per cent of plasma peak) in 3 to 5 hours. The peak activity per milliliter of packed WBC was reached in 7 days, and was 167 per cent of the plasma peak, when the total activity in all circulating white cells was 4 to 8 per cent of the administered dose.

*This method was found on trial to give good white cell yield, RBC : WBC = 40:60 in number.

†DNA reprecipitated from the N KOH solution with trichloroacetic acid (TCA) and dissolved in 5 per cent TCA at 90° C. showed equal activity as that of original N KOH solution, indicating that the small protein fraction in N KOH solution did not interfere with the result.

The variations in the relative activities in these different fractions of the whole blood agreed satisfactorily with each other. As the respective volumes of WBC, RBC, and plasma were determined from time to time, the activity per milliliter of whole blood could be estimated from the measured activities of RBC, WBC, and plasma. The following table shows the result of comparison of the activity per milliliter of whole blood estimated in this way with that obtained by direct measurement.

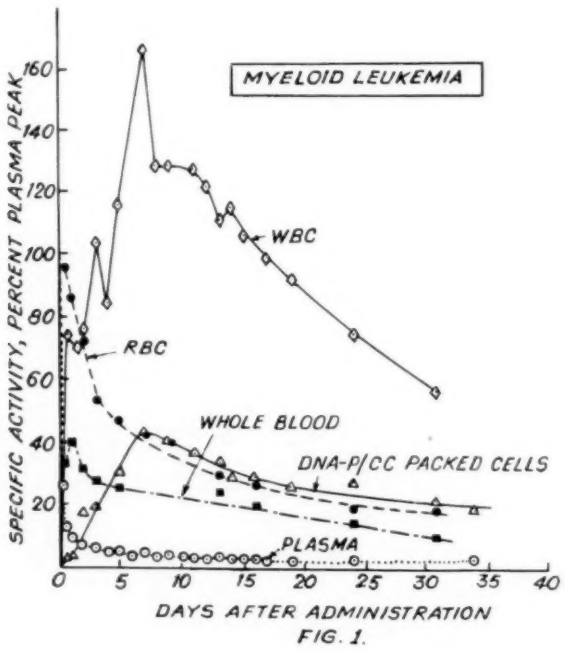

FIG. 1.

The activity in WBC was further analyzed into the activities of acid soluble phosphorus, phospholipids, RNA, and DNA phosphorus fractions. These results have been indicated in Fig. 3. In this figure the peak activity per milliliter of packed white cells has been normalized to 100 and the activities in acid soluble phosphorus fraction (ASP) per milliliter packed white cells, RNA, and DNA phosphorus per milliliter of packed white cells have been expressed as per cent of peak white cell activity. When the activities in the several fractions were added up, they agreed within experimental errors with the total activity per milliliter of packed WBC as shown by the dashed curve in Fig. 3.

It was found that the activity in the acid soluble portion of the WBC reached the peak within 12 hours after administration. The RNA curve showed a sharp rise within the first 12 hours. It continued to rise at a slower rate, and reached the peak on the seventh day. The growth in activity of DNA and phospholipids was of the same nature. The peak activity in phospholipids was reached in about 7 days, about the same time in which the

activity in the DNA fraction reached its peak. However, the DNA peak was higher than the phospholipid peak, and the activity in DNA was always greater than that in the phospholipid fraction. The peak DNA activity was about 25 per cent of the WBC peak (on the seventh day), after which the activity in DNA started to decrease slowly. Significant activity remained in the DNA fraction even 34 days after administration of P^{32}.

Fig. 4 shows the activity variations in different fractions of blood with days, in the subject with chronic lymphatic leukemia. The variations showed very slight differences from those in patients with chronic myeloid leukemia.

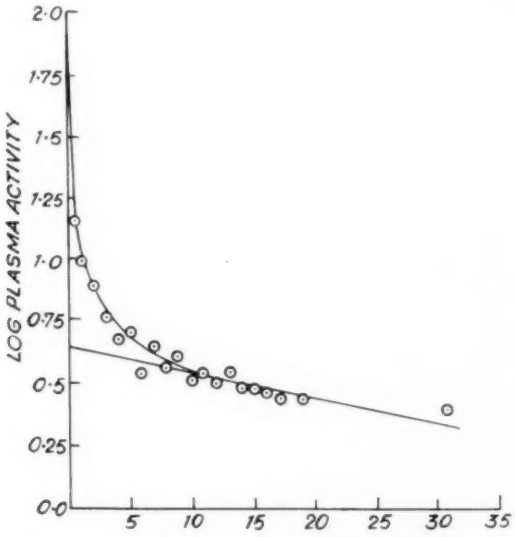

FIG. 2.

Peak activity of white cells on the seventh day was 130 per cent of the plasma peak. The relative activities in the different phosphorus fractions of lymphocytes were of the same nature as those in patients with myeloid leukemia (Fig. 3).

TABLE I. COMPARISON OF THE ESTIMATED AND MEASURED ACTIVITIES PER MILLILITER OF WHOLE BLOOD

	RELATIVE VOLUMES (ML.)			MEASURED ACTIVITIES PER ML. (IN % OF PLASMA PEAK)			ESTIMATED ACTIVITY PER ML. WHOLE BLOOD (% OF PLASMA PEAK)	MEASURED ACTIVITY PER ML. WHOLE BLOOD (% OF PLASMA PEAK)
DAYS	PLASMA	RBC	WBC	PLASMA	RBC	WBC		
0.5	1.85	0.7	0.45	14.0	96	71.5	40.0	34
3.0	2.00	0.7	0.30	6.0	52	104.0	27.4	28
7.0	1.15	0.6	0.15	4.0	40	167.0	28.0	24
16.0	1.12	0.64	0.16	3.0	29	103.0	20.0	19
24.0	1.12	0.7	0.18	2.5	22	74.0	15.8	14

Fig. 5 shows the DNA activities in the patients with myeloid and lymphatic leukemia plotted on a logarithmic scale. The log of the activity in DNA (as per cent of plasma peak) has been plotted in this figure. The DNA curve in lymphatic leukemia rose to a peak in 9 days (Figs. 4 and 5) and then decreased comparatively rapidly up to the eighteenth day, after which it became almost flat. The DNA in the subject with lymphatic leukemia also showed a relatively lower activity.

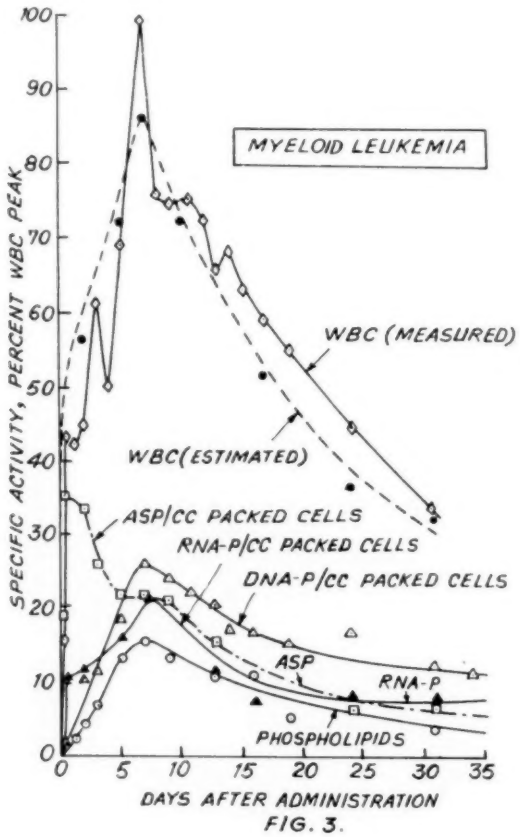

FIG. 3.

DISCUSSION

A study of the growth in activity of white cells and its different fractions shows that white cells have a good amount of activity in 12 hours and half or more of its peak activity by 24 hours. The major contribution is from the acid soluble phosphorus fraction (ASP) and next in order is that from RNA phosphorus. Very little activity is shown in DNA phosphorus in the first 24 hours but it reaches almost one fourth of its peak by 48 hours. From this it appears that within a short time inorganic phosphorus enters the ASP and RNA P fractions of white cells to be released into circulation in 12 hours. The pattern of uptake of phosphorus in different fractions suggests that ASP forms the immediate precursor pool for the other fractions.

It is seen from Fig. 5 that tagged myelocytes do not appear in circulation before 12 hours and that tagged lymphocytes do not appear before 24 hours. These are the minimum times required for the maturation of these cells within the bone marrow, including the short interval taken by radioactive phosphorus to enter the DNA precursor pool.

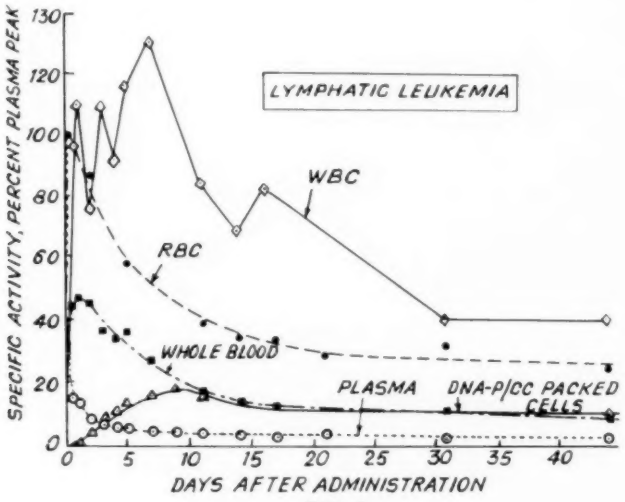

FIG. 4.

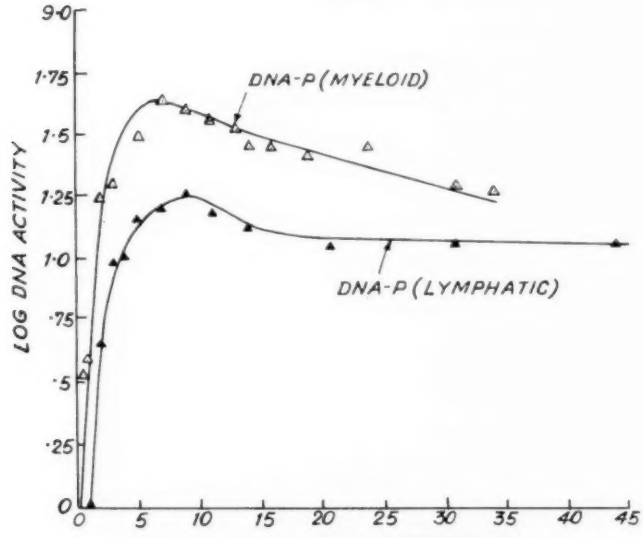

FIG. 5.

It is seen from Fig. 3 that activity in the acid soluble phosphorus in the circulating white cells reaches its peak value within as short a time as 12 hours. From this it may be concluded that the activity in precursor pool of DNA in the bone marrow reaches its maximum within a very short time after application of P^{32}.

Let P_o be the total number of P^{32} atoms which are uniformly mixed up with V_p atoms of phosphorus in the DNA precursor pool in bone marrow at the peak. Then the peak activity in this pool is given by

$$p_o = P_o/V_p \tag{1}$$

Thereafter the absolute number of P^{32} atoms in the precursor pool diminishes and that in the DNA pool increases. Neglecting the very short interval that is taken by the activity to reach its maximum in this precursor pool, one can express the decrease in the activity in the precursor pool with time by the equation

$$p = p_o\, e^{-r_1 t} \tag{2}$$

where p is the activity remaining after a time t, and r_1, the rate constant of decrease.

The increase in the number of radioactive atoms (Q) in the DNA fraction may then be represented by

$$\frac{dQ}{dt} = r_1 P - r_2 Q \tag{3}$$

where r_1P is the rate of increase of Q by the transfer of radioactive atoms from the precursor pool and r_2Q the rate of decrease of radioactive atoms in the DNA pool by their removal due to any process. The total number of P^{32} atoms present in the DNA pool at any instant (t) is given by

$$Q = \frac{r_1}{r_2 - r_1} P_o\, (e^{-r_1 t} - e^{-r_2 t}) \tag{4}$$

The activity in the DNA pool is

$$q = \frac{Q}{V_q} = \frac{r_1}{r_2 - r_1} \frac{P_o}{V_q} (e^{-r_1 t} - e^{-r_2 t}) \tag{5}$$

In the steady state, since there is no accumulation of phosphorus and the rate of loss of DNA-P from the circulation is equal to the rate of entry from the precursor pool, we have

$$r_1 V_p = r_2 V_q \tag{6}$$

With the help of equation (6) the activity in the DNA pool may be represented by

$$q = \frac{r_2}{r_2 - r_1} p_o\, (e^{-r_1 t} - e^{-r_2 t}) \tag{7}$$

where p_o is the maximum activity in the precursor pool. Equation (7) shows that $q = 0$ when $t = 0$; q then increases and reaches a maximum value at a time t_m given by

$$t_m = \frac{2.3}{r_2 - r_1} \log \frac{r_2}{r_1} \tag{8}$$

After reaching the maximum, q diminishes in a complicated manner depending on r_1 and r_2, the rate constants of the diminution of activity in the precursor pool, and circulating DNA pool.

It seems reasonable to assume that activity in the DNA precursor pool dies out at a much slower rate than the activity in the DNA of the circulating white cells, since the latter is strictly limited by the definite life of the white cells, while the former, only a minute fraction of the total phosphorus pool, is drawn for synthesis of DNA. If it is assumed that r_1 is small compared to r_2, in a sufficiently large time equation (7) reduces to

$$q = \frac{r_2}{r_2 - r_1} p_o e^{-r_1 t} \quad (9)$$

The decrease in activity of the DNA of white cells is then governed by the rate constant (r_1) of the precursor pool. The value of r_1 can be obtained from the slope of logarithmic plot of the activity in DNA with time, as shown in Fig. 5. Substituting the value of r_1 thus obtained and the observed value of tm, when the peak activity is reached in the DNA fraction, in equation (8), one gets the value of r_2. The average period of stay of white cells in circulation is given by $1/r_2$.

The rate constant of the decrease of activity of DNA after reaching the peak was deduced to be 0.035 day^{-1} from Fig. 5 in patients with myeloid leukemia. Taking the experimentally determined value of $t_m = 7$ days in patients with myeloid leukemia when DNA activity was maximum, r_2 was calculated from equation (8) to be 0.37 day^{-1} with the corresponding average life of 2.7 days. The cases under investigation had the circulating granulocytes composed of myelocytes and neutrophils in the ratio varying from 40:60 to 60:40. Hence the life determined is that of an average population. This result agrees with that found by Osgood.[11]

The pecularity of the DNA curve in subjects with lymphatic leukemia is possibly due to the presence of two groups of cells, one very long lived and other short lived. For the short lived group of cells, with $r_1 = 0.035$ day^{-1} and with the observed peak activity on the ninth day, one can deduce $r_2 = 0.265$ $days^{-1}$ from equation (8). This corresponds with an average life of 3.8 days.

If the circulating white cells represent a true cross section of the total white cell population, including those in extravascular spaces maintaining a continuous dynamic equilibrium, the periods found above represent the actual life of white cells, otherwise they represent only the time for which white cells stay in circulation.

SUMMARY

After application of a tracer-dose of radioactive phosphorus to 3 chronic myeloid and 1 chronic lymphatic leukemia subjects, the growth of activity in the different fractions of blood and white cell phosphorus was studied. The average periods of stay of leukemic leukocytes in prevascular and vascular phases were derived from the observed rates of variation of activity in the desoxyribose nucleic acid of circulating white cells. These periods were found to be 12 hours and 2.7 days, respectively, in the case of granulocytes, and 24 hours and 3.8 days for one population of lymphocytes.

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THE CLEAN-VOIDED URINE CULTURE IN SURVEYING POPULATIONS FOR URINARY TRACT INFECTION

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THE prevalence of asymptomatic urinary tract infection in a sampled hospital outpatient population is estimated at 5 per cent.[1] Since studies based on outpatient populations cannot be applied to the general population without reservations, estimates as to prevalence in the general population remain tentative. However, if asymptomatic urinary tract infection of this magnitude does exist in the general population, mass screening techniques might be warranted for purposes of detection and treatment.

The most generally used procedure for the detection of bacteriuria is the broth culture of a urine specimen. This approach is inadequate because the common occurrence of scant contamination during collection of the specimen yields an inordinately high number of false positive cultures. Since a common contaminant, *Escherichia coli*, is also the most common pathogen, the positive broth culture is difficult to evaluate and often must be repeated. This is time consuming and, moreover, hardly suitable for survey purposes when subjects may be available on only one occasion. Recent studies have amply shown the value of quantitative bacteriology (urine colony count) on fresh urine specimens in the differentiation of casual contamination from significant bacteriuria.[1-3] In the initial studies catheterized specimens were utilized for the quantitative bacteriology. Unfortunately, the unpleasantness of the procedure and the risk of introducing infection, estimated at 2 per cent,[1, 3-6] preclude catheterization in studies of the mass population. As an alternative to catheterization, Merritt and Sanford have shown that clean-voided urine specimens collected by nurses are entirely adequate for quantitative study.[5, 7] As part of a large scale epidemiologic survey of atomic bomb survivors being conducted at the Atomic Bomb Casualty Commission (ABCC) in Hiroshima, Japan, we tested the feasibility of male and female subjects collecting their own clean-voided specimens after instruction by nursing personnel. Furthermore, since colony counting requires much time, expense, and personnel, we tested the use of the Gram stain of the urinary sediment in determining which urines actually require the more elaborate procedure of colony counting on plated media.[8, 9]

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METHODS

Selection of Patients.—ABCC patients consist of atomic bomb survivors within 2,000 M. of the hypocenter and groups of individuals, suitable for comparison, who received presumably negligible or no irradiation. Urine specimens were taken without knowledge of the patient's radiation exposure classification from all hypertensive subjects and from roughly twice as many randomly selected nonhypertensive subjects during a 4 month period. The ages of the 441 patients studied range from 17 to 83 years with 95 per cent of patients between 30 and 69 years. No pregnant women were included.

Collection.—The purpose of the procedure was explained to the patient, after which he or she was instructed in the method of collection. After washing his or her hands with soap and water, the patient prepared for collection by cleansing the glans or vulva with a cotton ball soaked with 1:1,000 aqueous Zephiran. The meatus was then dried with a sterile cotton ball, and a midstream urine specimen was collected in a sterile, routine specimen jar. The collected specimen was covered and taken immediately to the laboratory, where inoculation of the cultures was carried out within a 2 hour period.

Bacteriologic Technique.—A 10 ml. aliquot was aseptically removed and placed in a sterile, conical centrifuge tube. After centrifugation at 3,000 r.p.m. for 15 minutes, the supernatant was decanted, and the sediment was studied as follows: (1) a portion was removed with a sterile platinum wire loop, placed on a slide, and a Gram stain was done; and (2) in all instances thioglycollate broth was inoculated with the sediment.

The quantity of urine plated for the colony count was determined by the number of bacteria seen in the Gram stain of the sediment. If "none" or "rare" bacteria were seen, appropriate dilutions in normal saline were made as follows: (a) "few," 1:10 dilution; (b) "many," 1:100 or 1:1,000 dilution. Counts were done on selective media for both gram-positive and gram-negative organisms. For gram-positive organisms a media which contained blood agar plus 0.02 per cent sodium azid (Difco B45) was used; for gram-negative organisms tergitol-7 agar (Difco B455) was used. The prepared plates were inoculated with 0.1 ml. of the appropriately diluted specimen, with the use of a sterile 1 ml. pipette. The inoculum was evenly spread by gently tilting the plate. When the plate had been incubated for 24 hours at 37° C., the colonies were counted with a Quebec colony counter. Counts were corrected to 1 ml. of urine and were reported separately for the selective media. When the count was under 10,000 colonies per milliliter, identification was usually made on the basis of colony characteristics and the Gram stain. In all cases of pure growth or where the count exceeded 10,000 colonies per milliliter the usual biochemical reactions were used to confirm the identification. In such cases the identification was checked against the original broth culture.

RESULTS

Colony Counts With Casual Contamination.—The problems of collection are greater in females than in males. Thus, the data are shown separately by sex in Table I and Fig. 1. It will also be seen that counts on contaminated specimens rarely exceeded 10,000 colonies per milliliter of urine. The counts exceeding 10,000 colonies per milliliter were 15,000 colonies per milliliter on two occasions and 20,000 colonies per milliliter on one occasion. Two were from diabetic subjects with glycosuria and the other was from an obese female with *Trichomonas vaginalis* infection.

The Gram Stain.—Table II shows the distribution in normal subjects of Gram stain reports on the urinary sediment as related to the final colony count. Many bacteria were seen in the sediments of only 3.5 per cent of urines from males but in 22.3 per cent of specimens from females. The nature of these bacteria are considered below.

Types of Contaminants in Urines With a Positive Gram Stain ("many").—Table III shows the distribution of the contaminating flora. Diphtheroids and *Staphylococcus albus* coexisted in the vast majority (86 per cent) and were the hallmarks of contamination. One or the other was present in combination with another contaminant in 93 per cent of the cases. *E. coli* and enterococci (not exhibiting beta hemolysin) made up the bulk of the remaining contaminants. In only one case was a count of less than 10,000 colonies per milliliter associated with a pure growth. This was a 9,000 colonies per milliliter count of *Aerobacter aerogenes* in a patient who, after panhysterectomy and irradiation for adenocarcinoma of the uterus, was shown to have considerable residual bladder urinary volume (120 ml.) at the time of our studies. A clean-voided specimen and a catheterized specimen done serially were almost identical (Patient 6 in Table IV).

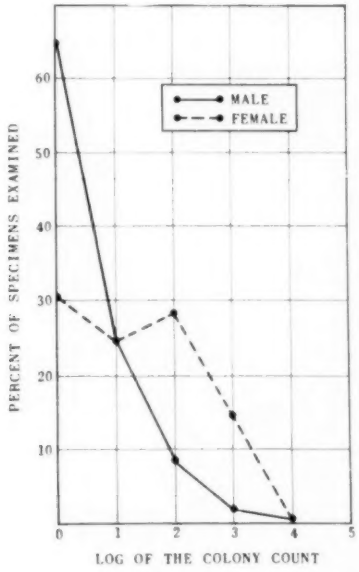

Fig. 1.—The frequency of urine bacteria counts plotted separately by sex.

Urinary Tract Infections in This Period.—Seven patients with significant bacteriuria were detected in the study period and were studied more extensively (Table IV). Ten to 116 days elapsed between the collection of the first and second clean-voided urine specimens. The findings conform with the experience that high counts remain high and that catheterized specimens subsequently yield counts of the same order of magnitude as the previous clean-voided specimen. High colony counts tend to yield pure growth although such counts were occasionally associated with the simultaneous isolation of *Staphylococcus albus* or a diphtheroid. All cases of true bacteriuria had the Gram stain reported as "many gram-negative bacilli" except in Patient 6 (Table IV) at the time that the colony count was 7,000 colonies per milliliter, and the Gram stain was reported as "few gram-negative bacilli." Counts of under 10,000 colonies per milliliter are consistent with a Gram stain report of "few" organisms.

DISCUSSION

Sanford and co-workers[2] showed that there was a significant shift in the flora isolated from urines with counts over 1,000 colonies per milliliter and considered that values over this indicated infection. Kass[1] felt that contamination yielded counts generally under 10,000 colonies per milliliter and that infection generally yielded counts over 100,000 colonies per milliliter. He indicated that there was an overlap in the range of 10,000 to 100,000 colonies per milliliter

TABLE I. DISTRIBUTION OF URINE BACTERIA COLONY COUNTS IN NORMAL SUBJECTS

LOG OF THE COUNT	MALES (NO.)	FEMALES (NO.)	TOTAL
0	131	72	203
1	50	57	107
2	17	65	82
3	4	33	37
4	1	4	5
5	0	0	0
Total	203	231	434

TABLE II. THE FREQUENCY IN NORMAL SUBJECTS OF VISIBLE BACTERIA IN THE URINE SEDIMENT AS DETERMINED BY THE GRAM STAIN

LOG OF THE COLONY COUNT	GRAM STAIN IN MALES				GRAM STAIN IN FEMALES			
	0-RARE	FEW	MANY	TOTAL	0-RARE	FEW	MANY	TOTAL
0	116	13	2	131	41	21	10	72
1	32	14	4	50	28	22	7	57
2	12	4	1	17	18	24	23	65
3	2	2	0	4	12	11	10	33
4	0	1*	0	1	1*	0	1	2
5	0	0	0	0	0	0	0	0
Total	162	34	7	203	100	78	51	229

The gram stain was not done in two female subjects.
*Diabetic patients.

TABLE III. NATURE OF THE CONTAMINANT IN URINE SPECIMENS IN WHICH THE GRAM STAIN REVEALED ABUNDANT BACTERIA

LOG OF COLONY COUNT	TOTAL CASES	DIPHTHEROID	STAPH. ALBUS	E. COLI	ENTEROCOCCI	AEROBACTER & KLEBSIELLA	PROTEUS	STAPHYLOCOCCUS AUREUS	PARACOLON	LACTOBACILLUS
0	12	10	11	5	7	0	0	0	1	1
1	11	10	11	4	3	0	0	0	1	0
2	24	23	24	11	7	0	0	2	3	0
3	10	8	6	8	5	4	2	0	0	0
4	1	1	1	0	0	0	0	0	0	0

(log 4). In the discussion that followed the presentation of Kass' paper, Sanford stated, ". . . We felt that numbers of bacteria somewhere around 10,000 were significant for infection. Actually . . . the number is somwhere between 10,000 and 100,000. . . ."[1] The current experience is in agreement with this conclusion, despite the fact that catheterization was not used in the collection of the specimen.

In considering the 7 infected patients detected, it should be noted that all were women over 49 years of age and that a thorough clinical history in 6 would have sufficed to alert the physician to the possibility of a urinary tract infection (Table IV). However, the Gram stain proved adequate in presaging significant counts in all cases. Thus, of the 64 cases in which the Gram stain revealed abundant ("many") bacteria, these were seen to be gram-negative bacilli in 6 instances, and all 6 subsequently showed persistent bacilluria on repeated culture. Of 172 cases classified as "none," "rare," or "few" in the Gram stain procedure 1 was subsequently shown to be infected. A 95 per cent

TABLE IV. URINARY INFECTIONS DETECTED DURING THE STUDY PERIOD

PATIENT	AGE	SEX	COLONY COUNT ON GRAM-NEGATIVE MEDIA	COLONY COUNT ON GRAM-POSITIVE MEDIA	GRAM-NEGATIVE ORGANISM	REMARKS
1. Mit	49	F	1,000,000	0	E. coli	Direct questioning revealed a history of recurrent cystitis
			484,000	0	E. coli	
			180,000(C)	0	E. coli	
2. Hir	61	F	500,000	1,000	E. coli	Patient had had previous bowel operation and gave a history of nocturia and low back pain
			1,000,000	0	E. coli	
3. Mor	69	F	500,000	0	E. coli	History of recurrent pyelonephritis and had had operation for retroflexed uterus
			*14,000	0	E. coli	
			10,000,000(C)	0	E. coli	
4. Iji	65	F	80,000	0	E. coli	History of acute pyelonephritis 20 years previous. IVP showed calcified pelvic mass deflecting the left ureter. Kidneys normal
			1,000,000	0	E. coli	
5. Sue	49	F	7,000	0	E. coli	Direct questioning revealed nocturia (3 to 4 times) "since youth" with daytime frequency but no dysuria
			100,000	0	E. coli	
			18,400,000	0	E. coli	
			5,350,000(C)	0	E. coli	
6. Miy	54	F	9,000	0	A. aerog.	Pelvic carcinoma, postoperative. Findings consistent with retention cystitis
			†10,240	0	Kleb. pn.	
			†11,560(C)	120	Kleb. pn.	
7. Suz	54	F	50,000	230	B. paracolon	No symptoms at time of routine examination
			1,000,000	0	B. paracolon	

The colony counts are listed in the order obtained.
Counts labeled (C) were obtained by standard catheterization techniques.
*Believed to have been treated by her private physician shortly prior to obtaining this specimen.
†Obtained serially.

confidence interval on 1/172 would have an upper limit of about 3.2 per cent. This is the estimate of error that mass use of the Gram stain seems to imply. Alternatively, one might say that 1 urinary tract infection in 7 would go undetected if the Gram stain alone were used. However, it is reasonable to

anticipate that the Gram stain, at best, agrees with the subsequent colony count. In such case, the apparent error (Patient 5) had a colony count (7,000 colonies per milliliter) below that regarded as diagnostic. This implies that Patient 5 did not have significant bacilluria *at the time* the Gram stain was done. If so, the above figures are better considered 6/64, 0/172, and 0/6.

Since a Gram stain classified as "many" was noted in only 3.5 per cent of males, this classification may justify further quantitative studies in males. However, in not a single one of these male subjects did the Gram stain reveal gram-negative organisms. In 4 cases, abundant gram-positive rods were noted and in the remaining 3 cases, gram-positive cocci. The Gram stain applied under these conditions in women was classified "many" in 22.3 per cent, and this classification would appear to be less efficient in females for screening purposes.

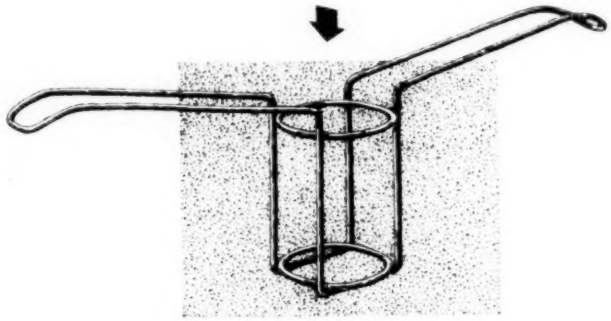

Fig. 2.—Apparatus for holding conventional urine specimen jar during collection of clean-voided urine.

Actually, contaminating organisms in the specimen were usually identified as gram-positive cocci (staphylococci) and club-shaped rods (diphtheroids). Thus, for screening purposes a urinary sediment exhibiting "many" gram-negative organisms may be considered presumptive evidence of infection. This is not meant to imply that only gram-negative organisms are pathogenic, but only that for screening nonhospitalized populations, this classification will suffice.

Contamination was not only discernible by the absolute count but by the nature of the isolated organism. Thus, the combination of *Staph. albus* and diphtheroids was virtually diagnostic of some degree of contamination. Commonly associated with either or both were *E. coli* and enterococci. In the case of true bacilluria with coincident but scant contamination, the use of counts on differential media sufficed to delineate the situation. In such cases the medium selective for gram-negative organisms yielded high counts compatible with infection and the medium selective for gram-positive organisms yielded no growth or a scant count consistent with contamination (Patients 2, 6, and 7).

Caution must be exercised in interpretation of results obtained with urine specimens of diabetic patients. First, it is conceivable that gram-positive organisms may more frequently be pathogenic in diabetic than in normal subjects. If this is the case, abundant gram-positive cocci in the urine sediment from a diabetic subject would not be so suggestive of contamination as it is in normal subjects. Our data offer no evidence for this. Rather, it should be pointed out

that the 2 diabetic subjects with counts exceeding 10,000 colonies per milliliter did *not* have abundant flora in the sediment (Table II). This finding is consistent with the alternatives that contaminants from diabetic patients contain a greater number of viable organisms or that glycosuria creates an optimal medium for contaminant growth in the period between staining and plating of the specimen. If the latter is correct, it underscores the urgency of immediate plating of urines from diabetic subjects.

At the termination of this segment of the study the nursing personnel were invited to make comments. They noted that patients unavoidably wet the outside of the specimen jar during collection and understandably found this aesthetically unpleasant when subsequently handling the jar. We successfully eliminated this by using the apparatus shown in Fig. 2 to hold the specimen jar during collection. Thus, during and after collection, the patient no longer had to hold the container, which instead was handled by a gloved nurse. The value of this simple modification is most appreciated by patients.

SUMMARY AND CONCLUSIONS

1. Clean-voided, midstream urine specimens were obtained from 441 ambulant, nonhospitalized subjects and were examined by means of the Gram stain and quantitative culture technique.

2. Collection was performed by the subjects themselves after appropriate instruction; this yielded specimens satisfactory for quantitative study.

3. The Gram stain of the urinary sediment proved efficient in presaging true bacilluria. The presence of abundant gram-negative organisms in a *fresh* urine sediment may be considered presumptive evidence of a urinary tract infection.

4. Utilizing prepoured, selective media, it was found that counts exceeding 50,000 to 100,000 colonies per milliliter indicate true bacteriuria and will be confirmed by repeated studies.

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STUDIES ON LIPOPHAGOCYTOSIS

I. Lipophagocytosis by Human White Blood Cells in Vitro and in Vivo

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THE mechanism by which chylomicra are removed from the blood and by which fat is deposited in the tissues has still not been elucidated completely. Two principal ways have been proposed: (1) hydrolysis of the neutral fat in the blood stream mediated through the lipoprotein lipase system, and (2) removal of the chylomicra as such, either through phagocytosis by the cells of the reticuloendothelial system, or by passage through pores in the capillary walls directly into the parenchyma, especially of the liver.[1, 2]

Evidence for phagocytosis of fat particles by the reticuloendothelial cells was presented in 1895 by Beneke,[3] who demonstrated the appearance of fat droplets in these cells after the injection of fat emulsions into the blood stream of the dog. Similar observations were made by Gilbert and Jomier[4] in 1908, and by Jaffé and Berman[5] in 1928. Marchand[6] observed finely dispersed sudanophilic granules in the reticuloendothelial cells in the liver, spleen, and bone marrow of a patient with diabetic hyperlipemia. Leary[7] found that in cholesterol fed rabbits a large number of fat droplets accumulated in the Kupffer cells of the liver. He further claimed that the fat-loaded Kupffer cells became detached from the sinusoids, entered the circulation, and deposited the fat in the tissues after they had penetrated the vessel walls. Byers, Mist-St. George, and Friedman[8] identified cholesterol in the Kupffer cells of cholesterol fed rats. Indirect evidence for chylomicron phagocytosis in vivo, based on the pattern of removal of chylomicra, has been presented by French and Morris.[9]

The idea that circulating leukocytes phagocyte fat was entertained at the beginning of this century by Anitschkow[10] and others in connection with the pathogenesis of atheroma formation. Beneke[3] showed that fat droplets, which lodged in the lung capillaries of dogs given intravenous injections of fat, were gradually phagocyted by leukocytes and were removed by them first into the perivascular tissues and then into the alveoli.

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In spite of the recognized fact, that leukocytes are able to take up particulate matter, little attention has been devoted thus far to a possible role of the leukocytes in the transport and deposition of fat. This is surprising since, in pathologic fat deposition, such as atheromata, the presence of fat-loaded cells, lipophages, is a consistent finding. The origin of these cells is as yet obscure. Some investigators consider them to be wandering tissue macrophages[11]; Leary[7] believes them to be mobilized Kupffer cells, whereas Anitschkow[10] considers them monocytes which originate from the circulating blood.

This investigation was undertaken with the aim of gaining information on the possible role of the circulating leukocytes in fat transport and fat deposition in physiologic and pathologic conditions. This article is concerned with fat phagocytosis by normal human leukocytes in vitro and in vivo. Subsequent reports will deal with similar studies of the leukocytes of normal and cholesterol fed rabbits.

MATERIALS AND METHODS

Siliconized glassware was used throughout.

Leukocyte Suspensions.—Venous blood was drawn over acid citrate dextrose (A.C.D.) solution (5:1) into conical 15 ml. centrifuge tubes and spun at 500 r.p.m. for 5 minutes at room temperature. The supernatant was pipetted off and respun at 2,000 r.p.m. for 30 minutes, and was stored at 4° C. The buffy layer was collected with a Pasteur pipette and introduced into glass tubes, 8 cm. long with a 1 mm. diameter; they were sealed at one end with paraffin and were covered with a rubber cap. The tubes were then spun at 2,000 r.p.m. for 15 minutes, and the supernatant plasma was discarded. The remaining sediment consisted of a thin white upper layer and a thicker lower layer of packed red cells. The white layer was separated by pushing the paraffin plugs through the glass tubes with a metal rod which fitted the lumen, and were then transferred to a test tube which contained 1 ml. buffered saline (Veronal buffer, pH 7.4). The white cell suspension thus obtained was washed twice with 1 ml. buffered saline, and the white cell button which remained after the last washing was used in the experiments immediately.

Lipid Emulsions.—Commercial olive oil,* lecithin,† further purified by Dr. B. Shapiro, and tristearin C.P.‡ were used. The lipids were added to either plasma or serum from the same donor, or to buffered saline and were shaken vigorously for 2 hours at room temperature.

Chylomicron-rich plasma was obtained from compatible donors 3 hours after a fatty meal, which consisted of two eggs, 40 Gm. butter, 300 ml. cream, and 200 ml. milk. To increase the chylomicron concentration, the lipemic plasma was spun in plastic test tubes at 22,000 r.p.m. in a Servall centrifuge for 30 minutes; the subnatant was then reduced by puncturing the test tube.

Olive oil was labeled with I^{131} according to a modified procedure of Beres and co-workers.[12] I^{131}-labeled chylomicra were obtained from blood drawn 3 hours after a fatty meal which contained I^{131}-labeled olive oil in varying amounts.

Phagocytosis in Vitro.—The leukocytes were resuspended in 1 ml. of lipid emulsion to a concentration of approximately 10,000 cells per cubic millimeter, and were incubated at 37° C. for 60 to 90 minutes while being gently shaken in a mechanical shaker. Drops of the

*Shemen Company, Haifa, Israel.
†National Biochemical Corporation, Cleveland, Ohio.
‡Fischer Scientific Company, Montreal, Canada.

incubation mixtures were removed from some of the test tubes at 5 to 10 minute intervals for phase microscopical observation. In addition, smears were stained with Sudan IV and Sudan Black B. In quantitative experiments, mixtures from other test tubes were transferred into the broad ends of Pasteur pipettes which had been sealed at the narrow tip by heat. To avoid breakage of the Pasteur pipettes, they were placed with their sealed narrow end in centrifuge tubes filled with Plasticine, and were then spun at 2,500 r.p.m. for 20 minutes. The supernatant and the fat layer were pipetted off, the end of the capillary which contained the leukocytes was cut off, and the leukocytes were washed out with buffered saline.

They were then rewashed 3 times with buffered saline, and were counted. The leukocyte sample was then frozen for chemical and isotope determinations.

Total esterified fatty acids (TEFA) were determined according to the method of Stern and Shapiro.[13] *Nonesterified fatty acids* (NEFA) were determined chemically according to Dole.[14] Radioactive measurements of NEFA in lipid emulsions and leukocyte suspensions were performed on extracts prepared according to the method of Borgström.[15] Radioactivity was measured in a well-type scintillation counter.*

RESULTS

A. *Lipophagocytosis by Leukocytes in Vitro.*—Experiments were performed with leukocytes obtained from healthy young male and female individuals and with plasma or serum from the same donors. Blood samples were obtained while the donors were fasting at 3, 6, and 24 hours after a fatty meal.

Leukocytes and plasma obtained from donors in a fasting state are referred to as "fasting leukocytes" and "fasting plasma," whereas those from donors with alimentary lipemia are referred to as "lipemic leukocytes" and "lipemic plasma," respectively.

1. *Lipophagocytosis by fasting leukocytes in fasting plasma–olive oil emulsion:*

Microscopical Observation.—Phase microscopic observations of leukocyte suspensions in fasting plasma–olive oil (4:1 v/v) emulsions showed marked fat phagocytosis by granulocytes and monocytes 10 minutes after incubation at 37° C. The number of fat droplets visible in the leukocytes increased with incubation up to 60 minutes. After 60 minutes, most of the leukocytes were loaded with fat droplets, and surrounded by a clear halo. The nucleus and the granules were pushed to one side of the cell (Fig. 1). The fat droplets in the leukocytes were of variable size, their diameter ranging from about 2 to 12 μ. The fat-loaded leukocytes continued to show vivid ameboid movement.

Extending the period of incubation to over 60 minutes did not seem to increase the number or the size of the fat droplets in the leukocytes. After 2 hours' incubation in the plasma–olive oil emulsion, the leukocytes showed signs of degeneration, i.e., swelling, loss of granules, absence of ameboid movement, and appearance of large vacuoles in the cytoplasm. In this respect they did not differ from leukocytes incubated simultaneously in plasma without olive oil or in saline.

Sudan stained smears, taken from the mixtures between 10 and 60 minutes' incubation, showed increasing quantities of sudanophilic droplets in the leukocytes.

*Model No. SC 46, Tracerlab, Boston, Mass.

After incubation of fasting leukocytes in fasting plasma–olive oil emulsion there was an increase in the TEFA content of the leukocytes from all donors studied (Table I).

Radioactive Determination.—Leukocytes separated immediately after suspension in plasma–I^{131}-labeled olive oil emulsion did not show radioactivity.

The data in Table II substantiate the microscopic observations and the results of the chemical determinations presented above.

TABLE I. TOTAL ESTERIFIED FATTY ACID CONTENT (μEq/10^6 W.B.C.) OF FASTING AND LIPEMIC LEUKOCYTES INCUBATED IN FASTING PLASMA–OLIVE OIL EMULSION FOR 60 MINUTES AT 37° C.

	LEUKOCYTES					
			AFTER FATTY MEAL			
	FASTING		3 HOURS		6 HOURS	
DONOR NUMBER	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
1	0.037	1.87	0.042	0.40	0.034	3.20
2	0.039	0.26	0.110	0.34	0.030	0.77
2	0.039	0.26	0.150	0.45	0.030	0.59
2	0.039	0.68	0.110	0.42	0.030	0.65
3	--	--	0.140	0.50	--	--
3	--	--	0.140	1.13	--	--
4	0.040	0.47	0.093	0.15	0.020	0.56
4	0.040	0.65	0.053	0.30	0.020	2.40
4	0.040	0.55	0.033	0.35	0.020	1.60
5	0.060	3.30	0.080	0.35	0.056	1.16
5	--	--	0.080	0.62	0.056	1.55
5	0.060	1.17	0.068	0.35	0.045	0.41
5	0.060	1.13	0.068	0.30	0.045	0.43
6	0.010	0.49	0.067	0.18	0.047	0.95
6	0.010	0.86	--	--	0.047	1.21
7	0.036	0.97	0.073	0.48	0.032	0.33
7	0.036	1.17	0.073	0.25	0.032	0.33
8	0.030	0.47	0.040	0.32	0.034	0.535
8	0.030	0.31	0.040	0.25	0.034	0.43

It is noted that with increasing specific activity of the plasma–olive oil emulsion of constant olive oil content (upper part of Table II), there was an increase in the radioactivity of the incubated leukocytes. Chemical determinations of TEFA in the tagged oil-plasma emulsions and in the leukocytes, after their incubation in these emulsions, showed that the specific activity of the fat in the leukocytes corresponded to that of the olive oil–plasma emulsion. This indicates that the leukocytes showed no discrimination in phagocytosis of iodinated and noniodinated olive oil.

It is further noted that with decreasing olive oil concentration of the emulsions (lower part of Table II), there was a decrease in fat uptake by the leukocytes, as demonstrated by a decrease in leukocyte radioactivity out of proportion to the decrease in the radioactivity of the plasma–olive oil emulsion.

2. *Lipophagocytosis by lipemic leukocytes in fasting plasma–olive oil emulsion:* It may be seen from Table I that the leukocytes obtained 3 hours after the fatty meal had a higher TEFA content than the fasting leukocytes, whereas the TEFA content of the leukocytes obtained 6 hours after the fatty meal had

TABLE II. FAT UPTAKE BY FASTING AND LIPEMIC LEUKOCYTES INCUBATED IN FASTING PLASMA-I^{131}-OLIVE OIL EMULSION FOR 60 MINUTES AT 37° C.

DONOR NUMBER	OLIVE OIL–PLASMA EMULSION		LEUKOCYTES							
			FASTING		AFTER FATTY MEAL					
					3 HOURS		6 HOURS		24 HOURS	
	V/V	COUNTS/ML./ MIN.	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
1	1:5	96,246	0	96	0	36	0	110	--	--
2	1:5	96,744	0	104	0	93	0	100	0	115
3	1:5	97,096	0	125	0	83	0	98	--	--
4	1:5	100,000	0	182	0	127	0	198	0	165
5	1:5	117,150	0	225	0	136	0	198	--	--
6	1:5	147,952	0	262	0	220	0	210	0	275
7	1:5	216,560	0	590	0	230	0	440	--	570
8	1:5	267,240	0	637	0	159	0	720	0	810
1	1:10	10,720,000	0	90,000	0	54,655	0	121,000	0	150,000
2	1:20	5,230,000	0	41,400	0	18,200	0	36,100	0	78,937
3	1:50	2,976,000	0	8,700	0	3,420	0	5,600	0	7,900
4	1:100	114,150	0	247	0	118	0	210	0	290
5	1:500	22,000	0	23	0	0	0	12	0	34

TABLE III. TOTAL ESTERIFIED FATTY ACID CONTENT (μEq/10^9 W.B.C.) OF FASTING AND LIPEMIC LEUKOCYTES INCUBATED IN LIPEMIC PLASMA*–OLIVE OIL EMULSIONS FOR 60 MINUTES AT 37° C.

	LEUKOCYTES							
	FASTING		AFTER FATTY MEAL					
			3 HOURS		6 HOURS		24 HOURS	
DONOR NUMBER	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
9	0.19	1.85	0.28	0.690	0.29	1.6	—	—
9	0.19	1.30	0.28	0.750	0.29	0.9	—	—
10	0.09	0.80	0.45	0.620	0.43	1.1	0.10	1.3
11	0.05	1.80	0.17	0.445	0.21	1.3	0.07	1.6
11	0.05	1.90	0.19	0.445	0.24	1.1	0.07	1.6
Average increment		1.39		0.31		0.9		

*Plasma obtained 3 hours after a fatty meal.

TABLE IV. FAT UPTAKE BY FASTING AND LIPEMIC LEUKOCYTES INCUBATED IN LIPEMIC PLASMA*–I^{131}-OLIVE OIL EMULSION FOR 60 MINUTES AT 37° C.

	OLIVE OIL–PLASMA EMULSION		LEUKOCYTES							
			FASTING		AFTER FATTY MEAL					
					3 HOURS		6 HOURS		24 HOURS	
DONOR NUMBER	V/V	COUNTS/ ML./MIN.	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
9	1 : 5	88,117	0	50	0	60	0	12	0	90
10	1 : 5	100,000	0	163	0	73	0	125	0	184
12	1 : 5	120,400	0	310	0	158	0	290	0	275
12	1 : 5	120,400	0	298	0	160	0	195	0	320
9	1 : 10	5,250,000	0	38,100	0	12,300	0	29,600	0	41,400
9	1 : 50	100,000	0	235	0	113	0	196	0	264
10	1 : 100	14,000	0	30	0	8	0	16	0	41

*Plasma obtained 3 hours after a fatty meal.

returned to about the original fasting value or was even lower. These data will be dealt with later in this paper.

Phase microscopic observations as well as Sudan stained smears of leukocytes obtained 3 hours after a fatty meal, revealed after incubation a marked diminution in fat uptake as compared to that by the leukocytes obtained from the same donors in the fasting state. This decrease in fat phagocytosis by

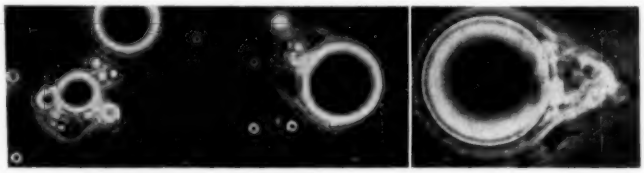

Fig. 1.—Phagocytosis of olive oil by leukocytes. Phase microscopical observation. (Left, ×900; right, ×1,200.)

lipemic leukocytes was substantiated both by chemical determinations (Table I, with 2 exceptions out of 18 experiments) and by radioactivity measurements (Table II). After incubation with plasma–olive oil emulsion, the TEFA content of the lipemic leukocytes did not reach, except in 2 cases, that attained by the fasting leukocytes of the same donor.

The leukocytes, obtained from these donors 6 hours after a fatty meal, showed a tendency to recover their capacity for fat phagocytosis (Tables I and II). In some cases, the TEFA uptake by these leukocytes was higher than that exhibited by leukocytes obtained from subjects in the fasting state.

Leukocytes obtained from subjects in the fasting state, 24 hours after the fatty meal, showed a fat uptake similar to that by the fasting leukocytes obtained on the previous day (Table II).

3. *Lipophagocytosis by fasting leukocytes in lipemic plasma–olive oil emulsion:* Morphologic observations as well as chemical and radioactive determinations on fasting leukocytes incubated in lipemic plasma–olive oil emulsion, yielded results comparable to those obtained with fasting plasma–olive oil emulsion. The results of the chemical and radioactive determinations are given in Tables III and IV and Fig. 2. Fig. 2 represents an experiment with fasting and lipemic leukocytes and plasma samples from the same donor. Both chemical and radioactive determinations were done on duplicate leukocyte samples obtained from the incubation mixtures both with fasting plasma (upper part of Fig. 2) and with lipemic plasma (lower part of Fig. 2) as medium.

4. *Phagocytosis of chylomicrons:*

a. Fasting leukocytes in lipemic plasma.—Fasting leukocytes incubated for 60 minutes at 37° C. in alimentary lipemic plasma obtained from the same donor were found to take up substantial quantities of fat, as observed under the phase microscope and as determined by chemical and radioactive methods (Table V).

The uptake of fat was lower than in the experiments with olive oil. The fat uptake by the leukocytes from chylomicron-rich plasma increased with

rising fat concentration and increasing specific activity of the plasma, which was similar to the results obtained in the experiments with olive oil emulsion. Hence, the lower fat uptake by leukocytes from the chylomicron-rich plasma may be explained by the latters' low fat content as compared with the plasma–olive oil emulsion.

b. Lipemic leukocytes in lipemic plasma.—Similar experiments on chylomicron phagocytosis performed with lipemic leukocytes showed a decreased fat phagocyting activity, as compared to that of fasting leukocytes (Table V).

c. Chylomicra suspended in fasting plasma.—In phagocytosis experiments with chylomicra obtained from a lipemic donor and resuspended in fasting plasma of the same donor, the leukocytes showed a fat uptake comparable to that in alimentary lipemic plasma. As in the experiments with olive oil, the fat uptake by the leukocytes in vitro was shown not to differ markedly, whether the emulsifying plasma was obtained from a fasting or a lipemic donor.

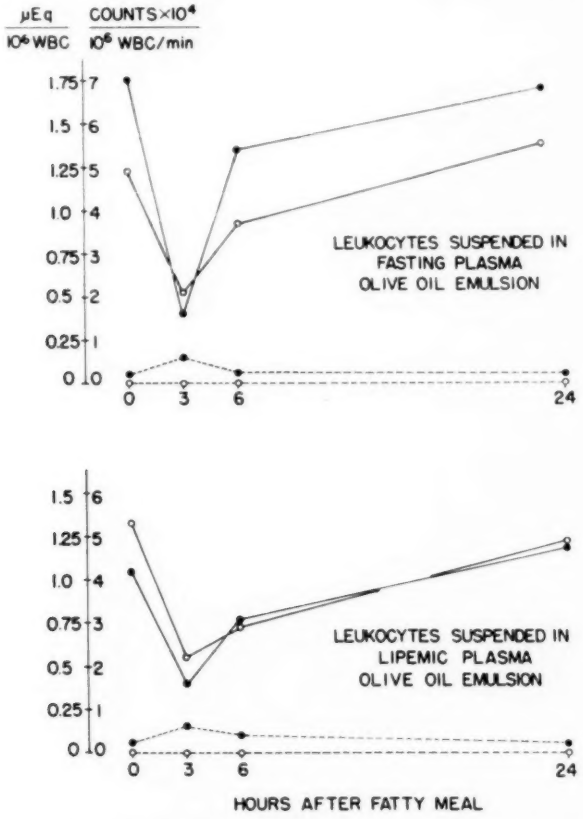

Fig. 2.—Fat uptake by fasting and lipemic leukocytes suspended in emulsions of olive oil in fasting plasma (upper part) or lipemic plasma (lower part).

5. *Phagocytosis of various lipids:* The uptake by fasting leukocytes of the following lipids suspended in fasting plasma was examined: poppy seed oil, tristearin, and lecithin.

TABLE V. FAT UPTAKE BY FASTING AND LIPEMIC LEUKOCYTES INCUBATED IN I^{131}-LABELED CHYLOMICRON-RICH PLASMA* FOR 60 MINUTES AT 37° C.

DETERMINATION	DONOR NUMBER†	CHYLOMICRON-RICH PLASMA	LEUKOCYTES							
			FASTING		AFTER FATTY MEAL					
					3 HOURS		6 HOURS		24 HOURS	
			BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
Chemical	1	9.3	0.030	0.49	0.080	0.17	—	—	0.036	0.53
μEq/ml.	3	11.9	0.040	0.91	0.070	0.32	0.056	0.49	0.038	0.98
or/10^6 W.B.C.	4	13.1	0.060	1.10	0.180	0.73	0.090	0.09	0.070	1.20
	5	12.3	0.035	0.87	0.073	0.42	0.040	0.81	0.041	1.00
Radioactive	1	272.0	0	17.00	0	7.00	0	14.00	0	26.00
counts/ml./min.	2	272.0	0	15.00	0	5.00	0	11.00	0	18.00
or/10^6 W.B.C./min.	3	690.0	0	57.00	0	23.00	0	26.00	0	54.00
	4	1,851.0	0	95.00	0	44.00	0	65.00	0	111.00
	5	1,427.0	0	71.00	0	22.00	0	39.00	0	59.00

*Lipemic plasma obtained 3 hours after a fatty meal which contained I^{131}-labeled olive oil.
†Corresponding donor numbers in the upper and lower part of the table indicate experiments on blood from the same donor.

Incubation with each of these 3 lipids led to an increase in TEFA content of the fasting leukocytes (Table VI). The leukocytes which had phagocyted poppy seed oil, tristearin, or lecithin contained fat droplets morphologically indistinguishable from those observed in the olive oil experiments.

TABLE VI. LIPOPHAGOCYTOSIS BY FASTING LEUKOCYTES IN FASTING PLASMA-LIPID* EMULSIONS

	LEUKOCYTES		
	TOTAL ESTERIFIED FATTY ACID CONTENT (μEq/10^6 W.B.C.)		
DONOR NUMBER	BEFORE INCUBATION	AFTER INCUBATION	
		Olive oil	Poppy seed oil
13	0.008	0.14	0.057
13	0.008	0.08	0.057
14	0.052	0.42	0.080
			Tristearin
14	0.052	0.37	0.150
15	0.029	0.58	0.081
15	0.029	0.29	0.058
15	0.029	0.465	0.058
			Lecithin
13	0.008	0.23	0.08
16	0.030	8.70	2.50
17	0.037	1.88	1.01
18	0.010	1.33	0.047

*Olive oil to plasma, 1:5 v/v; poppy seed oil to plasma, 1:5 v/v; tristearin to plasma, 100 mg./5 ml.; and lecithin to plasma, 80 mg./5 ml.

TABLE VII. FAT UPTAKE BY FASTING LEUKOCYTES INCUBATED IN FASTING PLASMA–OLIVE OIL EMULSION (V/V 5 : 1) OR SALINE–OLIVE OIL EMULSION (V/V 5 : 1) FOR 60 MINUTES AT 37° C.

	LEUKOCYTES (μEq/10^6 W.B.C.)		
		AFTER INCUBATION	
DONOR NUMBER	BEFORE INCUBATION	PLASMA EMULSION	SALINE EMULSION
20	0.17	2.80	1.80
20	0.17	3.90	2.10
21	0.08	0.30	0.33
21	0.08	0.36	0.30
22	0.031	5.80	2.60
22	0.031	12.00	3.00
23	0.28	1.85	1.45
23	0.28	1.30	1.60
24	0.45	1.43	2.00
25	0.11	0.68	0.64
25	0.11	0.68	0.95
26	0.21	0.86	0.82
26	0.21	--	0.76
27	0.32	0.90	0.90
27	0.32	1.15	1.02
28	0.34	2.50	2.00
28	0.34	2.50	1.50
29	0.22	1.23	0.98
29	0.22	--	1.30
Average increment		2.158	1.162

6. *Lipophagocytosis in serum and saline medium:* The fat uptake by leukocytes phagocyting in serum–olive oil emulsion was found to be comparable to

TABLE VIII. CHANGE IN NONESTERIFIED FATTY ACID CONTENT OF FASTING LEUKOCYTES INCUBATED IN FASTING PLASMA–I^{131}-LABELED OLIVE OIL EMULSION FOR 60 MINUTES AT 37° C.

		PLASMA–OLIVE OIL EMULSION WITHOUT LEUKOCYTES			PLASMA–OLIVE OIL LEUKOCYTE SUSPENSION				
					SUPERNATANT OF LEUKOCYTES	LEUKOCYTES			
		TEFA	NEFA		NEFA	TEFA		NEFA	
DETERMINATION	DONOR NUMBER	BEFORE INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
Chemical	1	83.4	0.13	0.70	0.935	1.25	3.50	0	0.176
μEq/ml. or/	9	427.0	0.43	0.61	0.840	1.83	3.70	0	1.160
10^6 W.B.C.	10	157.0	0.39	0.48	0.890		1.95	0	0.221
	13	183.0	0.61	0.94	0.940	1.38	2.74	0	0.930
	14	223.0	0.46	0.81	0.970	1.02	3.49	0.09	1.030
	15	--	--	--	0.370	1.20	3.90	0	1.770
Radioactive	1	96,700	2,400	4,200	5,800		92		38
counts/ml./	2	320,000	1,800	4,100	4,800		12,000		3,100
min. or/10^6	3	1,628	83	162	211		181		41
W.B.C./min.	4	100,000	3,600	4,700	8,900		482		87
	5	100,000	1,580	2,900	4,100		427		110
	6	25,660	298	395	481		425		190
	7	11,075	47	61	84		282		84
	8	97,700	1,114	1,666	2,100		1,590		483
	9	147,000	2,378	3,700	3,800		1,645		281
	11	83,000	416	341	427		341		41

TABLE IX. CHANGE IN NONESTERIFIED FATTY ACID CONTENT OF LIPEMIC LEUKOCYTES INCUBATED IN EMULSION OF PLASMA FROM FASTING SUBJECTS AND I^{131}-LABELED OLIVE OIL FOR 60 MINUTES AT 37° C.

DETERMINATION	DONOR NUMBER	PLASMA–OLIVE OIL EMULSION WITHOUT LEUKOCYTES			PLASMA–OLIVE OIL LEUKOCYTE SUSPENSION				
		TEFA	NEFA		SUPERNATANT OF LEUKOCYTES	LEUKOCYTES			
						TEFA		NEFA	
		BEFORE INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
Chemical	1	174	0.420	0.48	0.61	2.14	2.71	0.240	0.87
μEq/ml. or/	2	164	0.612	0.78	0.87	2.60	2.96	0.030	1.20
10^6 W.B.C.	3	374	0.580	0.95	0.95	2.15	3.41	0.158	1.94
	4	--	--	--	0.91	2.38	3.15	0	1.87
	5	214	0.400	0.84	0.89	2.30	3.70	0	1.10
Radioactive	1	100,000	2,900	4,100	5,000	--	74	--	36
counts/ml./	2	150,000	3,200	5,700	7,100	--	114	--	73
min. or/10^6	3	92,000	1,700	1,900	2,400	--	43	--	19
W.B.C./min.	4	225,000	4,300	7,000	8,400	--	265	--	145
	5	114,000	2,100	3,150	3,960	--	76	--	39
	6	63,000	1,920	2,240	3,000	--	93	--	60

TABLE X. PERCENTAGE OF NONESTERIFIED FATTY ACID OF TOTAL ESTERIFIED FATTY ACID IN FASTING LIPEMIC LEUKOCYTES INCUBATED IN PLASMA–I^{131}-LABELED OLIVE OIL EMULSION FOR 60 MINUTES AT 37° C.

	CALCULATED FROM TABLE VIII						CALCULATED FROM TABLE IX			
		PLASMA–OLIVE OIL EMULSION WITHOUT LEUKOCYTES		PLASMA–OLIVE OIL FASTING LEUKOCYTE SUSPENSION				PLASMA–OLIVE OIL LIPEMIC LEUKOCYTE SUSPENSION		
				SUPERNATANT OF LEUKOCYTES	LEUKOCYTES			SUPERNATANT OF LEUKOCYTES	LEUKOCYTES	
DETERMINATION	DONOR NUMBER	BEFORE INCUBATION	AFTER INCUBATION	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION	DONOR NUMBER	AFTER INCUBATION	BEFORE INCUBATION	AFTER INCUBATION
Chemical	1	0.3	0.8	1.00	0	4.8	1	0.4	11.0	32
	9	1.0	1.5	2.00	0	30.0	2	0.5	1.2	40
	10	0.2	0.3	0.50	0	12.0	3	0.2	8.0	57
	13	0.3	0.5	0.60	0	31.0	4	--	0	60
	14	0.2	0.3	0.48	9	28.0	5	0.4	0	30
	15	--	--	0.30	0	42.0	--	--	--	--
Radioactive	1	2.5	4.3	6.0	--	40	1	5.0	--	49
	2	0.4	1.3	1.5	--	25	2	4.7	--	64
	3	5.0	10.0	13.0	--	23	3	2.6	--	44
	4	3.6	4.7	8.9	--	18	4	3.7	--	55
	5	1.5	2.9	4.1	--	24	5	3.5	--	52
	6	1.1	1.5	1.8	--	45	6	4.8	--	75
	7	0.4	0.6	0.8	--	30	--	--	--	--
	8	1.2	1.6	2.1	--	30	--	--	--	--
	9	1.7	2.7	2.7	--	10	--	--	--	--
	11	0.5	0.4	0.5	--	12	--	--	--	--

that in plasma–olive oil emulsion. However, when saline was used as the emulsifying medium, the fat uptake by the leukocytes, although still appreciable, was in most cases considerably less than in plasma (Table VII).

7. *Changes in nonesterified fatty acid content of leukocytes during lipophagocytosis:* An effort was made to investigate the possibility of intraleukocytic breakdown of the phagocyted fat by determination of the relationship between the changes in the NEFA and the TEFA content of the leukocytes which occurred during lipophagocytosis.

Fasting and lipemic leukocytes were examined after phagocytosis of olive oil or chylomicra. The olive oil used in these experiments contained 0.5 per cent NEFA. The possibility of fatty acid liberation from the olive oil during the preparation of the plasma–olive oil emulsion was investigated. Examination of the subnatant plasma, separated from the plasma–I^{131}-labeled olive oil emulsion by centrifugation, however, revealed no radioactivity. Tables VIII and IX illustrate experiments in which TEFA and NEFA were determined in the leukocytes and in the plasma-fat emulsions. Table X summarizes the changes in NEFA to TEFA ratio which occurred during lipophagocytosis.

Plasma without leukocytes had a hydrolyzing activity as demonstrated by the increase in NEFA content of plasma-fat emulsions. In the presence of leukocytes the increase in NEFA content of the supernatant plasma-fat emulsions was somewhat more pronounced.

Before incubation, in most instances no NEFA could be detected in the fasting leukocytes; the lipemic leukocytes, however, generally contained a measurable quantity. After fat phagocytosis in vitro a relatively large proportion of the phagocyted fat could be accounted for as NEFA in both fasting and lipemic leukocytes, whether incubated in olive oil (Tables VIII and IX) or in chyomicron emulsion. Furthermore the data show that the NEFA to TEFA ratios in the lipemic leukocytes were higher than in the fasting leukocytes, although the total fat uptake by the former was less than by the latter (Table X).

In control experiments it was shown that leukocytes which had phagocyted I^{131}-labeled olive oil and which were resuspended in nonradioactive plasma did not release radioactivity into the medium during incubation.

B. *Lipophagocytosis by Circulating Leukocytes.*—

1. *After a fatty meal:* Blood was obtained from normal healthy subjects in the fasting state and 3, 6, and 24 hours after a standard fatty meal which contained I^{131}-labeled olive oil. Plasma and isolated washed leukocytes were examined for TEFA and NEFA content. Urine and stool were collected during 24 hours after the fatty meal, and their total radioactivity was determined and expressed as a percentage of the total administered radioactivity.

The data in Table XI show a consistent pattern of changes in the TEFA and NEFA content of the leukocytes. In every case there was a substantial increase in both TEFA and NEFA in the leukocytes 3 hours after the fatty meal. Six hours after the fatty meal the TEFA as well as the NEFA content

TABLE XI. FAT PHAGOCYTOSIS BY CIRCULATING LEUKOCYTES AFTER A I^{131}-LABELED FATTY MEAL

DETERMINATION	DONOR NUMBER		BEFORE		AFTER FATTY MEAL						PERCENTAGE OF ADMINISTERED RADIOACTIVITY EXCRETED IN 24 HOURS	
					3 HOURS		6 HOURS		24 HOURS			
			TEFA	NEFA	TEFA	NEFA	TEFA	NEFA	TEFA	NEFA	URINE	STOOLS
Chemical	1	W.B.C.	0.07	0	0.15	0.090	0.050	0	0.060	0.009		
μEq/ml. or/		Plasma	9.00	0.290	11.30	0.220	10.300	0.270	9.100	0.370		
10^6 W.B.C.	2	W.B.C.	0.05	0	0.17	0.080	0.056	0.036	0.056	0		
		Plasma	10.70	0.230	13.60	0.230	11.600	0.230	11.600	0.310		
	3	W.B.C.	0.08	0.001	0.21	0.110	0.080	0.009	0.077	0.007		
		Plasma	7.08	0.320	14.90	0.400	14.100	0.400	8.200	0.400		
	4	W.B.C.	0.036	0	0.11	0.072	0.030	0	0.033	0.006		
	5	W.B.C.	0.010	0	0.07	0.029	0.040	0.009	0.020	0		
	6	W.B.C.	0.010	0	0.65	0.030	—	—	—	—		
Radioactive	1	W.B.C.	0	0	48	48	15	12	8	0	13.0	0.27
counts/ml./		Plasma	0	0	555	28	415	21	14	0	—	—
min. or/10^6	2	W.B.C.	0	0	39	39	42	42	13	10	—	—
W.B.C./min.		Plasma	0	0	317	12	300	10	27	0	—	—
	3	W.B.C.	0	0	80	55	63	55	29	16	14.0	0.62
		Plasma	0	0	1,100	62	299	17	34	0	—	—
	4	W.B.C.	0	0	24	24	16	11	4	0	6.0	0.09
	5	W.B.C.	0	0	72	72	51	48	17	17	—	—
	6	W.B.C.	0	0	55	55	—	—	—	—	10.4	0.81

TABLE XII. EFFECT OF INTRAVENOUS HEPARIN* ON FAT PHAGOCYTOSIS BY CIRCULATING LEUKOCYTES

DETERMINATION	DONOR NUMBER	W.B.C. PLASMA	BEFORE		AFTER FATTY MEAL								PERCENTAGE OF ADMINISTERED RADIOACTIVITY EXCRETED IN 24 HOURS	
					3 HOURS		3½ HOURS		6 HOURS		24 HOURS			
			TEFA	NEFA	TEFA	NEFA	TEFA	NEFA	TEFA	NEFA	TEFA	NEFA	URINE	STOOL
Chemical μEq/10^6 W.B.C or/ml.	1	W.B.C.	0.030	0	0.780	0.066	0.84	0.200	—	—	—	—		
		Plasma	14.900	0.337	20.600	0.574	15.20	1.148	—	—	—	—		
	2	W.B.C.	0.015	0	0.930	0.096	0.98	0.210	—	—	—	—		
		Plasma	11.200	0.316	13.900	0.420	12.10	1.090	—	—	—	—		
	3	W.B.C.	0.033	0	0.114	0.040	0.23	0.098	0.06	0.042	0.021	0.008		
		Plasma	11.000	0.260	14.000	0.195	7.80	1.360	8.00	0.960	9.300	0.195		
Radioactive counts/10^6 W.B.C./min. or/ml./min.	1	W.B.C.	0	0	44	44	69	69	65	65	12	12	12.9	1.6
		Plasma	0	0	415	28	297	58	189	0	27	0	—	—
	2	W.B.C.	0	0	72	70	104	100	11	8	0	0	13.7	4.6
		Plasma	0	0	607	22	439	59	212	12	0	0	—	—
	3	W.B.C.	0	0	42	40	63	59	15	15	0	0	11.0	—
		Plasma	0	0	327	19	234	34	126	0	14	0	—	—

*Heparin, 200 mg. injected intravenously immediately after the 3 hour sample was drawn.

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of the leukocytes, as determined chemically, had returned in most of the cases to the fasting levels; the values after 24 hours were similar. Radioactive measurements of the leukocytes, however, showed after 6 and 24 hours substantial activity both of TEFA and NEFA. It is observed that, whereas in the chemical determinations the NEFA to TEFA ratio in the leukocytes 3 hours after the fatty meal was approximately 1:2, in the radioactive measurements it was approximately 1:1. The same ratio was found in the 6 and 24 hour leukocyte samples.

It is noted that at the time there was a marked increase in NEFA in the leukocytes, no such increase could be demonstrated in the plasma by chemical determination, and only a slight increase was detectable by radioactivity measurement.

2. *Effect of intravenous heparin:* The changes in TEFA and NEFA which occurred in the plasma and in the leukocytes of subjects with alimentary lipemia after intravenous injection of heparin are recorded in Table XII. It is seen that after heparin injection there was an increase of TEFA and NEFA in the leukocytes in all 3 subjects. These changes occurred when there was also a marked elevation of NEFA, but a decrease of TEFA, in the plasma.

COMMENTS

White blood cells capable of fat phagocytosis were polymorphonuclear leukocytes and monocytes, whereas the lymphocytes apparently did not participate in this process. In line with these observations is the finding that white cells, obtained from patients with chronic myeloid leukemia, showed a definite fat uptake in vitro, whereas leukocytes from patients with chronic lymphatic leukemia were practically devoid of fat phagocyting capacity. To our knowledge, fat phagocytosis by human white blood cells in vitro has not yet been described in the literature.

The ability of leukocytes, obtained from subjects with alimentary lipemia, to take up fat in vitro (olive oil or chylomicra) was considerably below that of leukocytes obtained from the same donors in the fasting state. The decrease in fat phagocyting capacity of the leukocytes persisted up to 6 hours after the fatty meal and returned to the original value after 24 hours. This difference in fat phagocyting capacity was determined by the state of the leukocytes and not by that of the suspending plasma medium, whether obtained from a donor in a fasting or alimentary lipemic state. Leukocytes obtained from donors with alimentary lipemia contained much more fat than those from donors in the fasting state. The diminished in vitro fat phagocyting capacity of the leukocyte from lipemic blood, however, does not seem to be due entirely to maximal in vivo fat loading, since in the phagocytosis experiments in vitro, their fat content never reached the levels attained by the leukocytes from fasting persons. No explanation for this phenomenon can be offered at present.

It should be pointed out that in all these experiments the chemical determinations reflect the increment of TEFA without providing information as to the dynamics of the fat originally contained in the leukocytes. On the other hand,

the radioactive determinations presumably reflect the uptake of fat from the medium, since it has been shown that radioactivity incorporated into the leukocytes was not liberated into the plasma during incubation.

In order to gain information on the fate of the phagocyted fat in the leukocyte, NEFA was determined in them as well as in the media. In our hands, when comparing the NEFA to TEFA ratios, the results obtained by the chemical method of Dole and the radioactive method of Borgström show a discrepancy; consistently higher ratios were obtained with the latter method. The cause of this discrepancy is not apparent. In spite of the quantitative differences in the results obtained with these 2 methods, their concurrent application revealed a similar qualitative pattern of changes which occurred in the NEFA in the leukocytes and in the plasma. In leukocytes obtained from subjects both in the fasting and in the alimentary lipemic states, there was a marked increase in NEFA after in vitro phagocytosis of olive oil as well as of chylomicra. Although there was a slight increase in the NEFA in the plasma–olive oil emulsion media incubated with or without leukocytes, the NEFA to TEFA ratios found in the leukocytes were much higher than those found in the plasma media. Any attempt to explain the relatively large quantities of NEFA in the leukocytes has to take into account the microscopically observed uptake of fat droplets by them. Hence, the most plausible explanation would be the intracellular hydrolysis of phagocyted fat. That leukocytes possess lipase activity has been shown by Bergel[16] and confirmed by Rossiter and Wong.[17] The possibility has not yet been excluded, however, that the above findings reflect a rapid uptake of NEFA by the leukocytes. This problem is being investigated.

The substantial increase in the fat content of circulating leukocytes and the appearance of radioactivity in them following ingestion of I^{131}-labeled olive oil seem to demonstrate that fat phagocytosis by leukocytes is not only an in vitro phenomenon, but also occurs in vivo. In vivo the NEFA to TEFA ratios in the leukocytes were very much higher than those in the plasma. The question as to where the fat phagocytosis takes place, whether in the circulating blood or extravascularly with subsequent re-entry of the fat-loaded leukocytes into the blood stream, cannot be answered at present.

It is surprising that, whereas phagocytosis of chylomicra by the cells of the reticuloendothelial system is considered to be possibly an important mechanism in their removal from the blood stream,[1] no mention is made in the pertinent current literature of the role the leukocytes might play in this process. The lack of information regarding the turnover of the fat in the leukocytes and the life span of fat-loaded leukocytes precludes the evaluation of the quantitative significance of leukocyte lipophagocytosis in fat transport. Using the observed average TEFA value of 0.15 μEq per 10^6 leukocytes, and assuming a blood volume of 5 L. and a white cell count of 10,000 per cubic millimeter, the amount of TEFA contained in the total circulating leukocytes at peak lipemia would be 7.5 mEq. On the other hand, taking at peak lipemia in the plasma a TEFA content of 12 μEq per milliliter and a hematocrit of 45 per cent, the total TEFA

in the circulating plasma would be 33 mEq. Thus the white cells would carry between one-fourth and one-fifth of the plasma TEFA load.

It is of interest in this connection to note the effect of heparin on leukocyte fat uptake in subjects with alimentary lipemia. After intravenous heparin injection, the TEFA content of the plasma diminished, whereas there was a concomitant increase in TEFA in the leukocyte. On the other hand, the NEFA rose both in the plasma and in the leukocytes, the increase being much more marked in the latter.

SUMMARY

Human white blood cells are capable of fat phagocytosis in vitro and in vivo.

The fat phagocyting activity of leukocytes was more pronounced in plasma than in saline medium.

The total esterified fatty acid content was higher in leukocytes obtained from alimentary lipemic subjects than in leukocytes obtained from the same donors in the fasting state.

The in vitro fat phagocyting activity of leukocytes obtained from subjects with alimentary lipemia was much less pronounced than that of the leukocytes obtained while they were in the fasting state. This difference was determined by the state of the leukocyte and not by that of the plasma medium.

The nonesterified to total esterified fatty acid ratios in the leukocytes after fat phagocytosis in vitro and in vivo, were much higher than those in the plasma, indicating intraleukocytic hydrolysis.

Heparin injection into alimentary lipemic subjects caused an additional increase in both nonesterified and esterified fatty acid content of the leukocytes, the rise being more marked in the former.

Quantitative considerations suggest that the circulating leukocytes play a significant role in fat transport.

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METABOLIC EFFECTS OF BACTERIAL PYROGENS UPON HUMAN LEUKOCYTES

I. Studies on Leukocyte Respiration, Glycolysis, and Nucleic Acid Synthesis

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THE mechanisms by which bacterial pyrogens induce fever have been the subject of many extensive studies.[1-3] A remarkable sequence of events has been postulated; it has been suggested that the fever results not from a direct action of bacterial pyrogen, but rather through the intermediary of damage to the host's leukocytes, which then release an endogenous pyrogen, the actual fever-evoking agent.[3-7] When bacterial endotoxin and leukocytes are incubated in a salt solution at 37° C., a reaction between them results in the disappearance of a heat stable pyrogen and the evolution in the medium of a heat labile pyrogen.[3] The biochemical processes by which bacterial pyrogen acts upon the leukocyte are little understood.

The following metabolic studies on the effects of bacterial pyrogens upon leukocytes were undertaken in order to understand more completely the leukocyte-bacterial endotoxin interaction. Pyrogens are virtually always present in laboratory materials and reagents; since separated leukocytes are now frequently studied in vitro, the need for definition of any unappreciated biochemical effects gave additional importance to the investigation.

Three parameters were examined: glycolysis, respiration, and nucleic acid synthesis. It was found that the pyrogens employed enhanced leukocyte glycolysis and, to a less extent, respiration, but exerted little or no effect on the incorporation of either a purine or pyrimidine into the nucleic acids of normal bone marrow and leukemic leukocytes.

METHODS AND MATERIALS

All glassware was siliconized and was rendered pyrogen-free by dry heat in an oven at 170° C. for 2 hours. All reagents were made up in water which had been triple distilled in pyrogen-free glassware.

Two bacterial products were used: Piromen, a polysaccharide derived from *Pseudomonas aeruginosa*, and a purified Shigella toxin.†

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†Provided by Dr. Leighton Cluff, Johns Hopkins University.

Three cell preparations were employed: bone marrow cells, and circulating leukemic leukocytes, suspended in the donor's own plasma anticoagulated with 0.02 mg. heparin per milliliter, and leukemic whole blood. The bone marrow samples were obtained from nonfebrile patients hospitalized for noninfectious disorders and were prepared for testing by gentle aspiration into and ejection from a syringe. Peripheral leukocytes from patients with untreated chronic myelocytic leukemia were separated from whole blood by sedimentation in an ice water bath. The nucleated cell count in the final suspension varied from one experiment to another, but was kept constant in each of the several preparations of a given experiment.

All studies were carried out in duplicate.

Leukocyte respiration was measured by a manometric technique.[8] Glycolysis was evaluated by determinations of lactic acid production over a 2 hour period at 37° C.[9] Screw capped 15 ml. culture tubes (16 by 125 mm.) were employed. For aerobic studies, room air was displaced with a mixture of 95 per cent O_2 and 5 per cent CO_2. For anaerobiosis a mixture of 95 per cent N_2 and 5 per cent CO_2 was used.

In the evaluation of nucleic acid synthesis, bone marrow aspirates and leukemic whole blood which contained 100,000 to 400,000 cells per cubic millimeter were used. Either orotic acid 6 C^{14} or adenine 8 C^{14} prepared in tris buffered isotonic saline* (10 μM per milliliter, S.A. 1 mc. per millimole†) was added in a final concentration of 2 μM of precursor per milliliter of cell suspension. The suspension then was transferred in 1 ml. volumes to each of a series of culture tubes, and 0.1 ml. of bacterial pyrogen was added in the concentrations noted in each experiment. Incubation was carried out, with constant agitation, at 37° C. for a period of 3 hours.

After incubation the suspension was washed once with ice-cold 0.85 per cent saline, and the red cells were hemolyzed with cold 3 per cent acetic acid. Cold 10 per cent trichloroacetic acid was used to precipitate the proteins and nucleic acids, from which the lipids were extracted with cold ether and ethanol.[10] The nucleic acids were then extracted with 10 per cent NaCl[11] in the cold at neutral pH, followed by extraction at 100° C. for 30 minutes. The sodium nucleates were precipitated with 2.5 volumes of cold ethanol. The ribose nucleic acid (RNA) was hydrolyzed with 0.1N NaOH at 37° C. for 24 hours,[12] then the desoxyribose nucleic acid (DNA) was reprecipitated in the cold with 2N HCl. Hydrolysis of the residual RNA in 0.1N NaOH at 80° C. for 30 minutes and reprecipitation of the DNA were carried out 3 times.

RNA and DNA were quantitated by the orcinol and diphenylamine reactions, respectively. Five-tenths milliliter aliquots of each nucleic acid solution were evaporated to dryness on stainless steel planchets, and their radioactivity was assayed in a thin window gas flow counter.‡ The results were expressed as counts per minute per 100 μg nucleic acid, at infinite thinness.

In some experiments, DNA bases were isolated. Desoxyribose nucleic acid was hydrolyzed in 12N perchloric acid in a boiling water bath for 1 hour. The hydrolyzate was then neutralized with 6N KOH. Ascending chromatography was performed on Whatman No. 1 filter paper with an isopropanol-HCl solvent.[13] The bases were eluted with 0.1N HCl, and quantitated according to the method of Bendich[14]; eluates of spots from adjacent areas served as controls. Samples for the determination of radioactivity were prepared as above, and the values were expressed as counts per minute per μM base.

RESULTS

Leukocyte Respiration.—Both Piromen and Shigella toxin enhanced respiration by separated leukemic leukocytes (Fig. 1). Piromen, both in concentra-

*Tris (hydroxymethyl) aminomethane 12.114 Gm. in 1,000 ml. of 0.8 per cent saline with HCl added to bring to pH 7.4.

†Volk Radiochemical Co., Chicago, Ill.

‡Nuclear-Chicago Corporation, Model D47.

tions of 0.1 and 1 μg per milliliter, induced an average increase of 22 per cent in oxygen utilization. In a concentration of 0.1 μg per milliliter Shigella toxin had very little effect, but 1 μg per milliliter induced increases of approximately 31 per cent and 46 per cent, respectively. Statistical analysis of these data revealed that the values are highly significant ($P < 0.01$). Differences between duplicate flasks did not exceed 10 per cent.

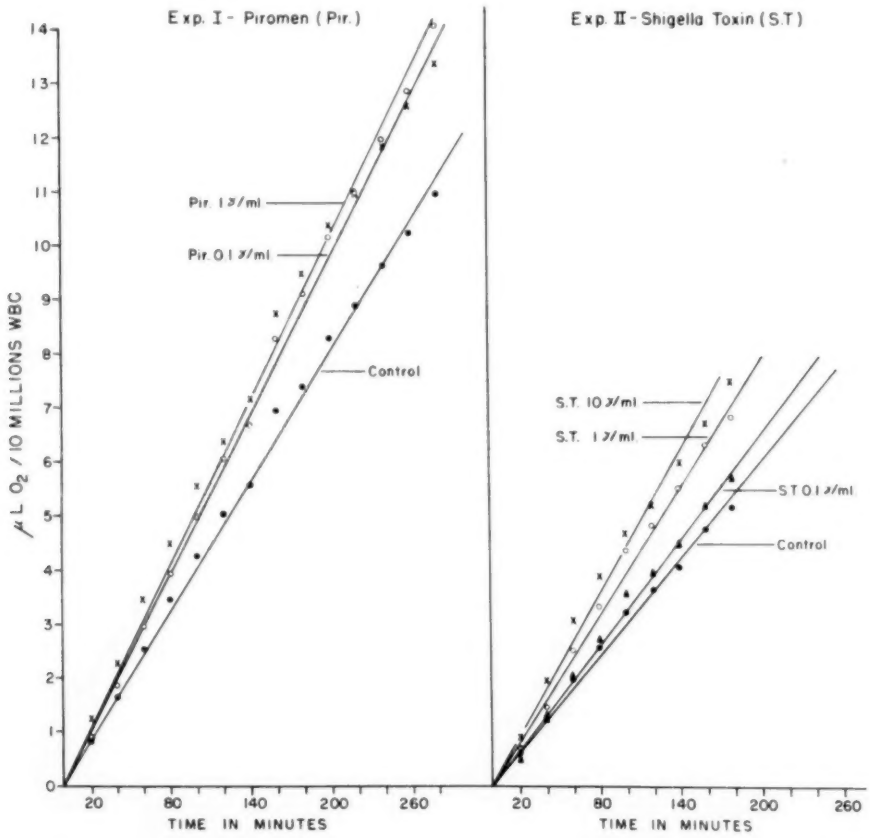

Fig. 1.—The effect of Piromen and Shigella toxin upon the oxygen uptake by leukemic leukocytes. Both experiments were performed on leukocytes from the same patient, although the samples were obtained on different days. A significant increase in the oxygen uptake was induced by both pyrogens ($P < 0.010$).

Glycolysis.—Both Piromen and Shigella toxin induced very marked increases in lactic acid production by leukemic white cells, whether separated or in whole blood. Piromen was much more potent; (Fig. 2) in concentrations of 1 μg per milliliter it increased aerobic glycolysis eleven-fold *(Exp. III)* and anaerobic glycolysis two- to sixfold *(Exp. III* and *IV)*. Shigella toxin in the same concentration was less active and in a concentration of 0.1 μg per milliliter had no effect *(Exp. II)*.

It was noted, in addition, that leukemic leukocytes produced more lactic acid anaerobically than aerobically. The converse was observed in 1 experiment

(Exp. I) carried out with leukocytes obtained from a patient with leukemoid reaction caused by liver disease.

Nucleic Acid Synthesis.—The results obtained with different cell preparations and nucleic acid precursors are as follows:

Orotic acid 6 C^{14}.—In Fig. 3, data are presented from a study with orotic acid as the precursor and both normal bone marrow and leukemic leukocytes as the cell preparation. Piromen and Shigella toxin in concentrations of 0.02 and 0.2 μg per milliliter did not affect the uptake of orotic acid into RNA or DNA.

Adenine 8 C^{14}.—Data in Fig. 4 fail to reveal any demonstrable effect of Shigella toxin, in concentrations of 0.01 and 0.1 μg per milliliter, on the incorporation of adenine into the DNA and RNA of normal bone marrow.

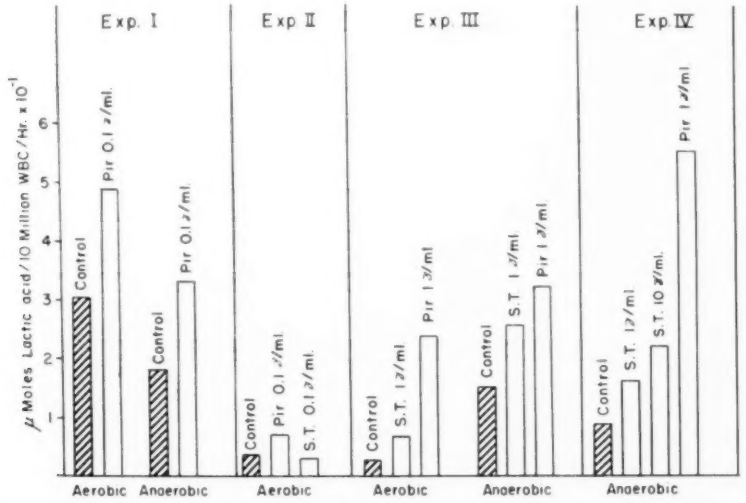

Fig. 2.—The effect of Piromen and Shigella toxin on aerobic and anaerobic glycolysis by peripheral leukocytes. *Exp. I*, Leukocytes from a patient with leukemoid reaction due to liver disease were studied. *Exp. II and III*, Leukocytes from one patient with chronic myelocytic leukemia were employed in 2 separate studies. *Exp. IV*, the same study was carried out with leukocytes from another leukemic patient.

Very marked increases in lactic acid production were brought about by both pyrogens in all experiments (except in *Exp. II* where Shigella toxin in a concentration of 0.10 μg per milliliter had no effect). Note also that more lactic acid was produced anaerobically by leukemic cells. The converse was true in *Exp. I* where nonleukemic leukocytes were studied.

When leukemic leukocytes were tested, the pyrogens may have influenced nucleic acid synthesis. The data are given in Figs. 5 and 6. In both experiments with Shigella toxin there was a 20 to 25 per cent increase in the incorporation of C^{14} adenine into RNA when the toxin was used in concentrations of 10 μg per milliliter. Piromen did not reproduce these effects and neither bacterial product clearly altered DNA synthesis. Furthermore, in 2 experiments with leukemic cells the DNA adenine and guanine were separated and their specific activities were determined (Table I). Both pyrogens, in concentrations of 1 and 2 μg per milliliter, had no evident effect on the uptake of radioactivity into the adenine and guanine bases.

Rabbit bone marrow was tested because of the susceptibility of this species of animal to Shigella toxin. In a single study, concentrations of toxin ranging from 0.001 to 1 μg per milliliter had no effect on the incorporation of C^{14} adenine into the nucleic acids (Fig. 7).

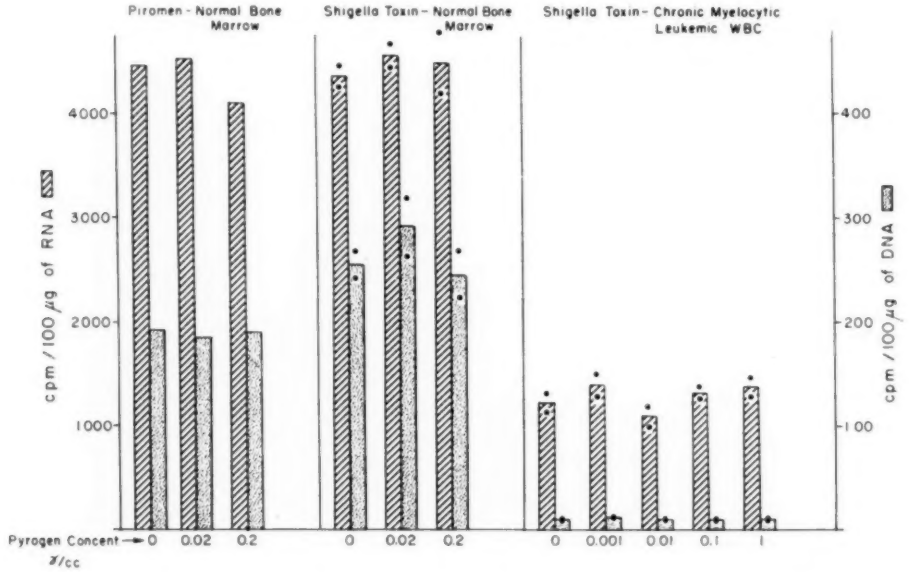

Fig. 3.—The effect of Piromen and Shigella toxin on the incorporation of orotic acid 6 C^{14} into the nucleic acids of normal marrow cells and circulating white blood cells of a patient with chronic myelocytic leukemia. The normal marrow cells were obtained from a single donor and tested simultaneously with both pyrogens.

No significant effect of Piromen was observed on the uptake of the precursors into the RNA or DNA. Similarly Shigella toxin did not influence the incorporation of orotic acid into RNA and DNA of either normal bone marrow or leukemic leukocytes.

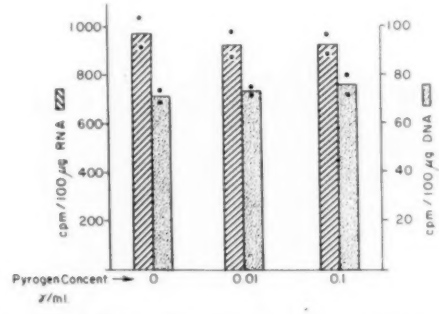

Fig. 4.—The effect of Shigella toxin on the incorporation of adenine 8 C^{14} into the nucleic acid of normal bone marrow. In concentrations of 0.010 and 0.10 μg per milliliter no effect was observed.

DISCUSSION

There have been many studies on the effects of bacterial pyrogens upon human leukocytes. Glycolysis and respiration and in vitro injury as determined

by the release of lysozyme have been especially investigated.[15-18] Martin and coworkers[16] reported marked increases in aerobic glycolysis in human leukocytes with decreases in oxygen consumption in response to endotoxin from *Neisseria intracellularis*, *P. aeruginosa*, *Salmonella typhosa*, and *Serratia marcescens*. The effects were elicited with concentrations of 0.005 to 0.5 μg of the endotoxins. Pneumococcal polysaccharide in the same concentration had no effect, but the carbohydrate of Type B *Klebsiella pneumoniae* stimulated oxygen uptake. The same authors reported that Piromen caused increases in lactate

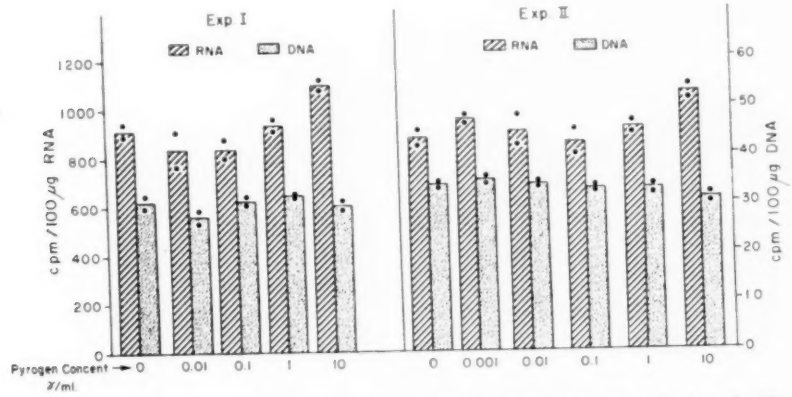

Fig. 5.—The effect of Shigella toxin on the incorporation of adenine 8 C^{14} into the nucleic acids of peripheral white cells of a patient with chronic myelocytic leukemia. Both experiments were performed with leukocytes obtained from the same patient on 2 separate occasions. Suggestive increases in the uptake of the precursor into RNA occurred in both experiments when Shigella toxin was used in a concentration of 10 μg per milliliter.

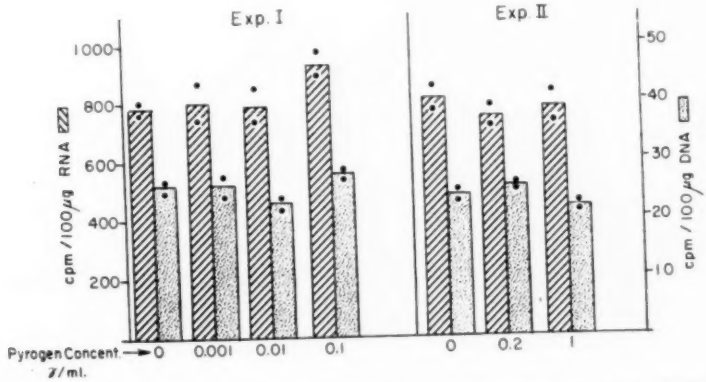

Fig. 6.—The effect of Piromen on the incorporation of adenine 8 C^{14} into the nucleic acids of peripheral white cells of a patient with chronic myelocytic leukemia. Leukocytes from the same patient were used at different times. No consistent effects on the uptake of C^{14} adenine into RNA or DNA were noted.

production when added in concentrations of 0.03 μg per milliliter, while toxin from *Corynebacterium diphtheriae* did not influence respiration or glycolysis.[17] It was apparent, therefore, that a difference in pattern was produced by various agents.

Kerby[18] has reported that variations in the degree of injury may be observed when leukocytes from different normal individuals of the same species are tested. In addition Alonso[19] studied the effects of pneumococcal products on glycolysis and respiration by the polymorphonuclear leukocytes of different

TABLE I. OBSERVATIONS ON INFLUENCE OF SHIGELLA TOXIN AND PIROMEN ON INCORPORATION OF ADENINE 8 C^{14} INTO DNA ADENINE AND GUANINE OF LEUKEMIC CELLS*

EXP. NO.	PYROGEN CONCENTRATION (γ/ML.)		C.P.M./100 γ DNA	C.P.M./μM DNA ADENINE	C.P.M./μM DNA GUANINE
1	Control	—	17	152	30
	Piromen	1	18	141	35
	Shigella toxin	1	15	135	36
2	Control	—	24	242	65
	Piromen	2	21	226	65
	Shigella toxin	2	22	266	81

*No significant effect was noted.

species. He found that in rat leukocytes, glycolysis was inhibited by purified Type 1 pneumococcal polysaccharide. Similar inhibition was not observed in the leukocytes of the guinea pig. It is of interest to note that the rat is susceptible and the guinea pig resistant to pneumococcal Type 1 infection. Results of a similar nature were reported in a study on the effect of bacterial fractions on the adenosine triphosphatase activity of nuclei-free homogenates obtained from guinea pig and rat leukocytes.[20]

In our studies, both Piromen and Shigella toxin enhanced respiration and glycolysis by leukemic leukocytes. In the smallest concentrations employed, Piromen was more effective. The finding of greater lactic acid production by chronic myelocytic leukemic leukocytes under anaerobic conditions is in agreement with the results reported by McKinney and Rundles,[21] who also compared both normal and leukemic leukocytes. It is of interest that the converse obtained in one experiment carried out with leukocytes from a patient with a leukemoid reaction due to liver disease, i.e., lactic acid production decreased under anaerobic conditions.

From the data on the action of bacterial pyrogen on nucleic acid synthesis by human leukocytes it would appear that, at most, only a minimal stimulation of the uptake of C^{14} adenine into RNA took place when concentrations of Shigella toxin as high as 10 μg per milliliter were used. No similar effects were noted with reference to DNA. When bone marrow from a rabbit (an animal highly susceptible to the pyrogenic effects of Shigella toxin) was employed, there was no demonstrable effect of the pyrogen on the incorporation of C^{14} adenine into either RNA or DNA.

The DNA incorporation of labeled precursors in peripheral leukocytes was relatively small, thereby introducing a possible limitation to interpretation of the results. However, when separation and determination of the radioactivity of the adenine and guanine bases were carried out, the results corroborated those obtained with whole DNA.

It must be recognized that while the experiments were designed to study the effects of pyrogens on nucleic acid synthesis by human leukocytes, there may have been pyrogen in the control system, since the radioactive precursors themselves might not have been truly pyrogen-free. Hence the findings presented may reflect only the effects of increments of pyrogen.

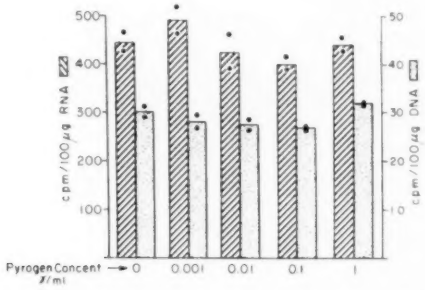

Fig. 7.—The effect of Shigella toxin on the incorporation of adenine 8 C^{14} into the nucleic acids of rabbit bone marrow. In concentrations of 0.001 to 1.0 μg per milliliter the pyrogen did not influence the uptake of C^{14} adenine into either RNA or DNA.

SUMMARY

Experiments were designed to study the effect of two pyrogens, a *Pseudomonas aeruginosa* polysaccharide and a Shigella toxin, upon glycolysis, respiration, and nucleic acid synthesis by normal bone marrow cells and circulating leukemic leukocytes.

Both the Pseudomonas polysaccharide and Shigella toxin enhanced glycolysis and oxygen uptake by leukemic leukocytes. The Pseudomonas polysaccharide was the more effective.

There was also a suggestive increase in the uptake of C^{14} adenine into the ribose nucleic acid (RNA) fraction of leukemic leukocytes when high concentrations of Shigella toxin were used. But in lower concentrations Shigella toxin had no effect on the incorporation of C^{14} adenine into the RNA and desoxyribose nucleic acid of rabbit bone marrow, and neither pyrogen influenced the incorporation of orotic acid into nucleic acids.

From the data obtained it may be concluded that the toxic effects of bacterial pyrogens upon leukocytes devolve primarily on metabolic processes other than those concerned with nucleic acid synthesis.

We are indebted to Dr. David Miller for his assistance in some of the studies on leukocyte respiration.

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LABORATORY METHODS

FOUR MODIFICATIONS OF THE MICRO AGAR DIFFUSION PRECIPITIN TEST

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SEROLOGIC tests in which antigen and antibody diffuse toward each other through agar gel and react to form specific precipitate bands are being applied analytically with increasing frequency in clinical and biologic investigations. To most of the virtues of classic precipitin tests in aqueous media they add their own advantages of high versatility, simplicity, and economy of time and materials. Many forms of agar diffusion precipitin tests have been developed since they originally were popularized by Oudin[1] and Ouchterlony[2] scarcely a dozen years ago. One which should prove to be outstanding is a method for their minification recently described by Wadsworth.[3] Not only does this technique require very small quantities of reactants, but its sensitivity and precipitin band resolution clearly exceed those of the usual macro tests.[3-5] An adaptation of Wadsworth's method to microscope slides for added convenience in storage of original reactions and for elimination of the need for apparatus not found in all clinical laboratories was recently reported.[5] This article shows how the principle of feeding reactants into extremely thin and uniform agar layers on microscope slides can be used quantitatively, describes a plastic matrix for a particularly versatile and sensitive comparator setup, and presents a microimmunoelectrophoretic method which requires only ordinary laboratory equipment but yields excellent results.

MATERIALS AND METHODS

Two antigen-antibody systems were employed. For single- and double-diffusion tests guinea pig antiserum was used against both bovine gamma globulin (BGG) and ovalbumin (OVA).* It was produced by a single subcutaneous injection of these 2 proteins in a water/oil tuberculolipid adjuvant emulsion 4 months before the serum was drawn. Immunoelectrophoresis was performed with a rabbit antiserum, against tuberculoprotein, similarly produced, although antigen injections (crude tubercle bacillary constituents) were given over a longer period of time before bleeding. The tuberculoprotein was an unrefined ammonium sulfate precipitate prepared in our laboratory from Kirchner synthetic culture medium supporting H37Ra tubercle bacillary growth.

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Procedures for setting up, for washing and staining, and for preserving permanently each of the micro diffusion tests mentioned below, were essentially those described previously.[5]

Agar and antigen for the experiments were dissolved in water buffered at pH 7.4 with sodium barbital.* Matrixes for single- and double-diffusion techniques were made of ⅛ inch thick Plexiglass, and those for immunoelectrophoresis from 1/16 inch Plexiglass. For the former 2 techniques, matrixes were 1 inch square, and for the latter they were slightly smaller in length and breadth than a standard microscopic slide. Well dimensions varied as indicated below.

RESULTS

Agar diffusion precipitin tests are most frequently used for qualitative analyses and comparisons of antigen-antibody systems. Quantitative analyses have received less recognition, study, and application than they deserve. Two examples of how micro quantitative single- and double-diffusion tests can be utilized are presented. They are adaptions of macro tests described by Feinberg.[6]

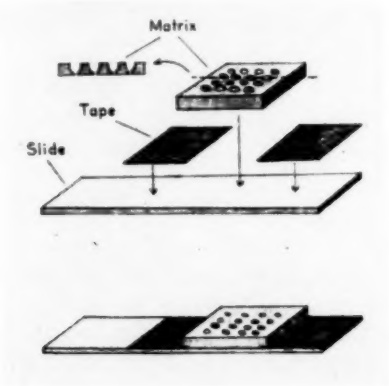

Fig. 1.—Sketch representing the method of setting up microscopic slide agar diffusion precipitin tests. The 16-hole matrix used for single-diffusion quantitative analysis is illustrated.

In addition to illustrating how slide micro diffusion tests are set up, Fig. 1 shows the type of matrix used for the single-diffusion quantitative method. This matrix has 16 identical double-diameter holes in rows of 4; the smaller diameter in contact with the agar is 1/16 inch and the larger is 9/64 inch. The matrix is laid upon agar solution, containing antiserum, pipetted at 56° C. onto the slide between supporting waterproof tapes. As soon as the agar has gelled, each of the 16 holes is filled with successive dilutions of antigen. The slide is then placed in a Petri dish and its precipitin reactions are allowed to develop for 24 hours in the refrigerator. Although these reactions can be observed immediately after the matrix is pried off, agar cloudiness caused by incorporated antiserum does not permit accurate readings until the agar has been washed free of reactants and the precipitates have been stained.

*Single- and double-diffusion tests buffer solution: 0.6 Gm. sodium barbital and 8.5 Gm. sodium chloride dissolved in 1,000 ml. distilled water and adjusted to pH 7.4 with approximately 2.1 ml. of 1.0N HCl; immunoelectrophoresis buffer solution: 7.2 Gm. sodium barbital in 1,000 ml. distilled water adjusted to pH 7.4 with approximately 14 ml. of 1:10 glacial acetic acid (ionic strength 0.023).

The reactions are read in terms of the first antigen dilution incapable of forming a precipitate disk against a given dilution of antiserum in the agar. This gives a ratio of antigen to antibody at which the antigen is presumably prevented from expanding into the agar by continual reaction and neutralization with equivalent amounts of antibody.

Although, in a sense the ratio of antiserum to antigen at which this occurs resembles the optimal proportions ratio of classic tube tests, fundamental differences between the 2 types of tests do not allow their respective ratios to be used interchangeably. Thus, in the classic test reactants are mixed together all at once, but in the single-diffusion agar precipitin test they are not. The effects of antigen and antibody reactions depend considerably on whether these reactants are mixed together piecemeal or all at once. In the classic test in which ungelled medium is used the optimal reacting ratio of antigen to antibody remains fairly constant if, for example, continually greater dilutions of antibody

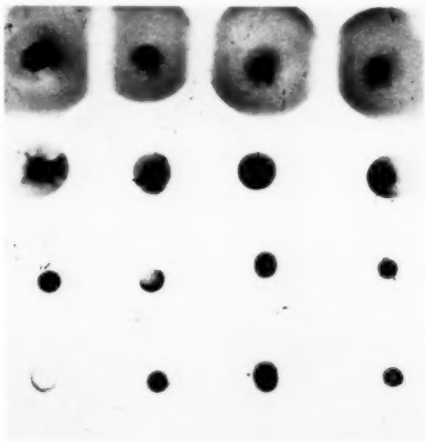

Fig. 2.—Results of a single-diffusion quantitative test in which a series of doubling dilutions of BGG, starting at 2 per cent, was reacted with 1:5 guinea pig antiserum in the agar. Antigen dilutions progress from left to right.

are added to a series of antigen dilutions.[7] This relationship holds true until antibody becomes insufficient to give a visible precipitate with its optimal antigen ratio. However, in the micro agar single-diffusion precipitin test the ratio is fairly constant only for a very narrow range of low antiserum dilutions, a point which must be remembered in the interpretation of the test. This technique should be used, then, primarily to compare antigen concentrations in unknown mixtures against a standard potent antiserum, although it could also be used to compare low dilutions of various antisera against a given battery of antigen dilutions.

Fig. 2 shows results from an experiment in which this technique was used; several doubling dilutions of BGG, starting with a 2 per cent solution in the upper left well, were reacted against a 1:5 dilution of antiserum. The first well to show no reaction, the ninth, contained 0.0078 per cent BGG, and, therefore,

the equivalence ratio could be expressed in terms of $\frac{\text{per cent antiserum}}{\text{per cent antigen}}$ as 2,564. The higher the original antigen concentration in an unknown solution, the higher proportionally this ratio will be, and vice versa. With a somewhat less rigid meaning, the higher the original antibody reactivity in an unknown antiserum, the lower this ratio will be.

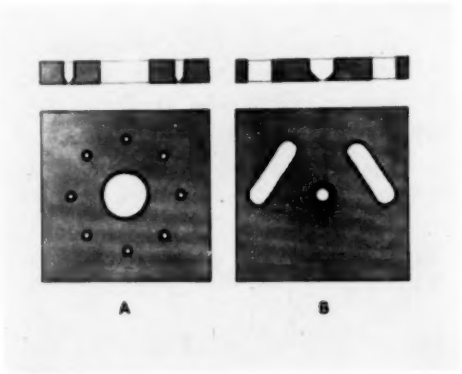

Fig. 3.—Face and cross-section diagrams of the micro double-diffusion quantitative test matrix (*A*) and the micro comparator matrix (*B*).

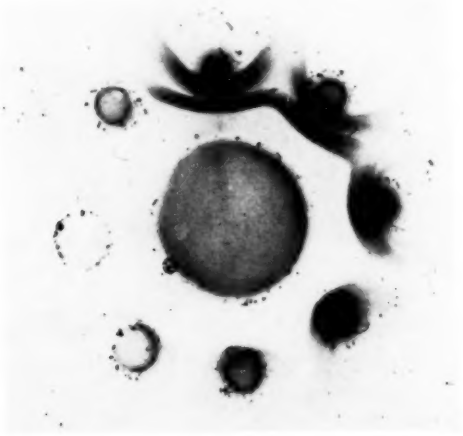

Fig. 4.—Reactions in the micro double-diffusion quantitative test of undiluted guinea pig antiserum, in the large central depot, against serial doubling BGG and OVA dilutions starting, at the top, with 0.03 per cent and progressing clockwise.

Double-diffusion agar precipitin tests also can be used to quantitate antigen and antiserum reactivity, and are particularly useful for simultaneous multiple system analyses. Fig. 3, *A* shows the type of matrix needed for such a test. This matrix has a central well with 1 diameter of $\frac{3}{16}$ inch and 8 satellite wells placed around it at equal distances, each with an upper diameter of $\frac{3}{32}$ inch and a lower diameter of $\frac{1}{16}$ inch. This double diameter helps prevent agar from

being drawn unequally up into these wells when the matrix is placed upon the warm agar solution. For most consistant results, agar should also be prevented from rising into the central hole. This is done by (1) very lightly waterproofing the inside of this hole with a water-resistant grease (e.g., silicone stop-cock grease), (2) using just enough agar to make uniform contact between the matrix and glass slide, (3) cooling this agar almost to the gelling point before pipetting it onto the slide, and, as an emergency measure, (4) temporarily sealing all the holes in the matrix from the top with a piece of transparent tape (e.g., Scotch tape) to create an equal air barrier to the agar in each hole. The tape can later be stripped off without moving the matrix from the gelled agar if the slide is chilled for 5 minutes in a refrigerator and the matrix, rather than the slide, is held against the pull of the tape as it is removed.

Fig. 5.—Micro comparator test with undiluted guinea pig antiserum diffusing from the central hole, 0.06 per cent OVA from the left slot, and 0.06 per cent each of OVA and BGG from the right slot.

Progressive dilutions of antigen in the satellite wells are tested against 1 concentration of antiserum in the central well. Fig. 4 illustrates results from this type of test in which successive doubling dilutions of BGG and OVA, both initially at 0.03 per cent concentration, were reacted against undiluted antiserum. Reactivities (quantities required for precipitation) of each constituent of both globulin and albumin are determined simultaneously. Two BGG fractions and an OVA fraction react through 0.0038 per cent but not at 0.0019 per cent; the second OVA component reacts through 2 greater dilutions to 0.00095 per cent but not at the next dilution. The meaning of these quantities depends upon the standard being used and specific experimental conditions (viz., well distances), as will be discussed below.

Perhaps the most popular application of agar double-diffusion precipitin tests in plates has been comparative antigen analyses in which not only the number of precipitin bands is observed but also their identity or nonidentity relationships are studied by their crossing, spurring, or coalescence upon

encountering each other. Although many well arrangements for micro tests could be devised as mere copies of those developed for macro tests and without any marked advantages over those already described,[3-5] it seems worthwhile to record here one which is particularly useful in overcoming the considerable disadvantage of reactant diffusion from point sources (i.e., circular holes in the matrixes) into continually expanding circular patterns. With this new matrix, the reference reactant is still allowed to diffuse from a circular hole, but the

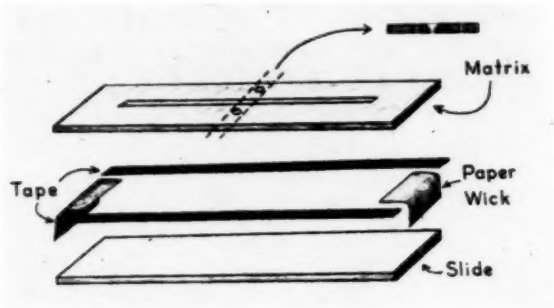

Fig. 6.—Sketch representing the matrix and arrangement of microscope slide components in microimmunoelectrophoresis.

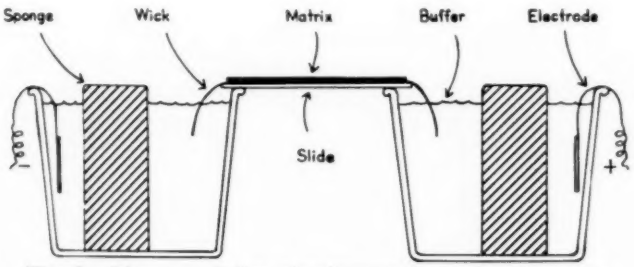

Fig. 7.—Diagram of the microimmunoelectrophoretic setup.

compared reactants are made to converge on each other and the reference reactant in uncurved or only convex fronts by being fed into the agar from 2 slots set at an angle to each other as shown in Fig. 3, *B*. In the matrix used for antigen comparisons (Fig. 5) these slots were ¼ inch long and 1/16 inch wide; the hole had a lower diameter of 1/16 inch and an upper diameter of 9/64 inch. As Fig. 5 demonstrates, excellent resolution can be obtained when this matrix is used.

Despite their versatility, single- and double-diffusion precipitin tests are sometimes applied to problems that cannot be solved without the aid of an outside analytic agent. One such agent which has proved itself amply since it was first applied to such problems by Grabar and Williams[12] is electrophoresis. Agar electrophoresis combined with agar diffusion precipitin techniques into immunoelectrophoresis as a powerful research tool undoubtedly tempts many

experimenters in biologic and clinical laboratories; it could also become an indispensible analytic tool to be used routinely in such laboratories, if existing techniques were simpler and required less specialized apparatus. The simplification of this tool described here makes it scarcely more difficult to apply than ordinary paper electrophoresis.

Fig. 6 shows that the basic piece of apparatus for this microscopic slide immunoelectrophoretic technique is a simple Plexiglass matrix, similar in form and purpose to those used for single- and double-diffusion tests, with a long central trough and 2 holes in its center on either slide of the trough. Other equipment, with the exception of the power source which many laboratories already will have on hand for paper electrophoresis, is simple and inexpensive. As Fig. 7 illustrates,[8] it consists of 2 synthetic sponges cut to fit across 2 buffer vessels and a pair of platinum electrodes with connecting wires. The test is set up as follows.

Two triple layers of tape are laid parallel lengthwise along the edges of the glass slide; between them at either end are laid filter paper wicks (Fig. 6). The type of filter paper used is unimportant as long as it is thinner than the combined layers of tape and is of a "quantitative" grade (e.g., Whatman No. 1). About 1.3 ml. of melted agar is spread over the slide with a pipette, and the matrix is lowered upon it and the tapes. The combination of slide, wicks, agar, and matrix is chilled in a refrigerator for about 5 minutes. Suction through capillary tubing slightly narrower than the holes in the matrix is used to remove agar down to the slide itself, and the solution to be electrophoresed is

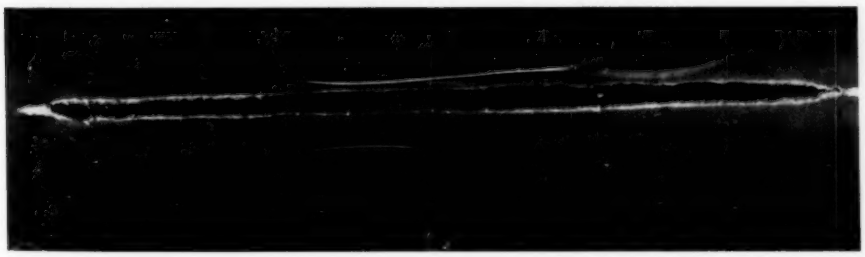

Fig. 8.—Microimmunoelectrophoresis: two 0.002 ml. samples of 2 per cent tuberculoprotein placed in their respective holes were submitted to electrophoresis for 50 minutes at 170 volts in a pH 7.4 sodium barbital buffer of 0.023 ionic strength, and were then allowed to react with undiluted rabbit antiserum placed in the central trough for 24 hours. The resulting reaction bands were stained with Thiazine Red R.[5] This is a direct projection print in which the stained slide was used as a negative and its enlarged image was printed on high contrast photographic paper.

placed in the resulting wells. Only about 0.002 ml. is required since the depth of the sample should be the same as that of the agar under the matrix. During electrophoresis the wells and the trough should be covered with transparent tape to prevent evaporation and local agar gel drying. The trough should be taped before the matrix is placed on the melted agar to prevent excess agar from rising into it; however, if this happens, the agar can be removed later by suction.

For electrophoresis the slide is rested as a bridge across opposing edges of the two buffer vessels with its paper wicks well immersed in the buffers (Fig. 7). Buffer levels in the 2 vessels should be equal, and, for the most efficient flow of

electric current, nearly high enough to touch the bottom of the glass slide. Fig. 8 shows results from a typical experiment and suggests the experimental conditions required. It also shows the advantage of using 2 antigen wells at different distances from the central trough in resolving individual fractions present in high concentrations together with other fractions of much lower concentration.

DISCUSSION

The clear advantages of micro agar diffusion precipitin tests over their macro predecessors, particularly in their higher sensitivity (resolution) and much greater economy, justify the description here of some practical modifications of the previously described microscopic slide technique[5] which should find use in clinical and biologic research. Certain points regarding their application will be discussed.

Some of the limitations and potential uses of the agar single-diffusion precipitin test have already been mentioned. A short résumé of how this test works should explain these more fully. Its basic principle, worked out with the Oudin single-diffusion tube technique,[9, 10] is that when equivalent concentrations of antigen and antibody face each other across their interface, a specific precipitate will form there; as this happens neither reactant will diffuse beyond the interface being neutralized locally by reaction. However, the ratio of equivalence at which this occurs in terms of original reactant quantities may not be the same as an analogous ratio arrived at with one of the classic tests in liquid media.

First let us consider reactions of serially diluted antigen against a constant but strong antibody concentration in the agar. At the antigen concentrations high enough to form disks of specific precipitate, all antibody in the immediate area of the antigen's diffusion source is combined with and neutralized so that additional antigen diffuses through the agar in this area unimpeded by reaction with uncombined antibody. Toward the edge of the expanding precipitate disk, however, free antigen concentration falls rapidly as the antigen is diluted both by diffusion and by combination with antibody until its rate of diffusion to the reaction front is matched by diffusion of antibody into that area from the surrounding agar, at which point the disk stops expanding. As the original source of antigen becomes more dilute, in the series of dilutions being used, the relative power of antibody in the agar to restrict antigen diffuson increases, and final disk diameter diminishes until it does not expand beyond the interface. Where this condition is attained, the ratio of antibody to antigen may be considered dynamically equivalent at the interface.

As we have mentioned above, the equivalence ratio observed with a low antiserum dilution in the agar (e.g., a 1:1 dilution) appears to change as higher dilutions (e.g., 1:5 dilution) are used. This change probably is due in part to the effect of agar in impeding reactant movement. Fig. 2 shows that otherwise perfect disks of precipitate formed by the first 4 antigen dilutions appear to be mutually dissolved or prevented from forming in areas where their respective influences overlap. Apparently, as the disks of precipitate expand, the larger

irreversible antigen-antibody reaction constituting them depletes its immediate area of antibody and draws more from adjoining areas; that is, a front of depleted antibody advances ahead of the front of visible precipitate. Thus, where the disks of precipitate grow toward each other, they create between them an area which lacks sufficient antibody to form any precipitate; in this area their edges will be incomplete. If the antigens which overlap were not identical, the disks would be entire and would overlap.

A similar condition near the source of antigen diffusion apparently arises measurably if the agar contains rather dilute antibody. As antigen begins its diffusion from the interface it combines with antibody in that area, depletes it, and "draws" more from the surrounding agar, in effect lowering that area's concentration. This occurs at all antibody concentrations in the agar, but since rate of fresh antibody movement into the depleted area depends on its original concentration in the agar, the more dilute it is, the farther the antigen (and its disk of precipitate) can advance against this potential movement before its front equilibrates with antibody. The result is that the apparent ratio at which antigen is held at its source with higher antibody concentration shifts in favor of the antigen at lower antibody concentration and is, therefore, constant only at a single antibody concentration. Thus, we have found that approximately half the antigen concentration predicted as the last able to form a visible precipitate disk on the basis of results with 1:1 antibody in the agar will produce a visible disk against 1:5 antibody. This does not limit the method's relative quantitative usefulness but calls attention to the fact that apparent "optimal proportions ratios" may vary in this and in any other test employing diffusion of reactants through agar so that only under certain conditions (e.g., high antibody concentrations) is this ratio similar to that of the classic precipitin tests in liquid media. The faster antigen diffuses in a particular experiment or technique, the greater will be this effect. Thus, if a high molecular weight antigen were employed or if the antigen were itself incorporated in agar, the effect could be suppressed or, in some instances, altogether eliminated.

The quantitative micro double diffusion agar precipitin test should be useful principally in the simultaneous measurement of the reactivity of numerous antigens occurring in widely diverging concentrations in their original mixture, in which this type of test is superior to the single-diffusion test because of its better resolution of minor antigen components (cf., Fig. 4). Results should be expressed relative to a selected standard of antigen or antibody because they depend simultaneously on several different factors, themselves relative (e.g., antigen molecule size to antigen concentration, antibody concentration, well sizes, and distances). For example, the quantity of an antigen in a solution being examined can be expressed as a multiple or fraction of a standard known quantity of the same antigen by observation of the extent to which the unknown can be diluted before reactions are extinguished and referral to experiments with known quantities of antigen to find the lowest concentration which forms a precipitate.

Within rather narrow technical limits, antiserum reactivity can also be quantitated in terms of what dilution of the unknown antiserum is needed to

create the same precipitin line pattern as a known antibody concentration against a series of antigen dilutions, with respect to distances of these lines from central or satellite depots. For example, referring to Fig. 4, we see that the 2 BGG components just touch the rim of the third well but not the rims of Wells 1 and 2. That is, antibody concentration was great enough to prevent, by precipitation, diffusion of antigen from the depot with a 0.0075 per cent antigen concentration, but not from depots containing 2 and 4 times this concentration. On the other hand, the OVA precipitates formed by the highest antigen concentration (Well 1) are about halfway between central and satellite wells. By contrast, when the same antiserum was used at a 1:4 dilution, the OVA lines of Well 1 formed tangents to the central depot since the antibody was insufficiently concentrated to hold them further away, and the BGG lines first touched a satellite at the 0.0034 per cent antigen concentration (Well 4). In an unknown serum, anti-BGG or OVA activity could be estimated by adjustment of the serum's concentration to match these precipitate patterns, and the factor of adjustment could then be used to calculate relative reactivity.

Technical alterations are possible on various aspects of this method. For example, a considerably finer determination of end points could have been made for the antigen-antibody systems than was used in the example given above by the use of smaller dilution steps. Since the Plexiglass patterns are so easy to make, extensive studies on any particular precipitating system could be preceded by varying hole sizes and distances to obtain a combination which suits it best.

The excellent precipitin band resolution of comparative analyses in which the micro agar diffusion precipitin technique is used is illustrated in Fig. 5, which also demonstrates the importance of such resolution. If one is able to ascertain the relative directions of the precipitin band tips, one can decide whether they are artifacts caused by Liesegang's periodic precipitation phenomenon or represent distinct antigen-antibody reactions. If they diverge, as they do in Fig. 5, they are not artifacts.

The essential feature of the Plexiglass matrix is its ability to cause all reactants to diffuse into a common area in such a way that precipitin bands tend to curve around the reference reactant well, an achievement of the trough-hole arrangement shown. Specific trough dimensions, sizes, or separating distances are factors which can be varied, within a moderate range, to optimally fit the antigen-antibody system under study.

Immunoelectrophoresis probably has more potential applications in biologic research than paper electrophoresis, which has become so common a tool, but its use is still restricted because current techniques, including microscopic slide methods,[11] require either too much experience or special apparatus to appeal to the nonimmunologist as an everyday analytic technique. We hope that the simplicity and effectiveness of slide microimmunoelectrophoresis described here will help reverse this situation.

In preliminary experiments with reactants of unknown concentration and complexity or in general survey work, the use of 2 holes set at differen

distances from the central trough for the solution being electrophoresed is a time- and trouble-saving device for the simultaneous detection of individual reactions against numerous components of widely varying reactivities. Thus, what otherwise might be construed as an imperfectly separated component by its formation between the trough and the nearer hole of a long, flat precipitate band with no clear apex really could be due to an excessive concentration of this component, something that will be shown on the side of the trough with the more distant hole, where this component becomes more dilute by diffusion before it reacts (cf., Fig. 8). On the other hand, some of the weaker electrophoretically separated components must be close to the reactant trough lest by diffusion they be diluted beyond their abilities to form visible precipitates.

We have mentioned previously[8] the use of an acid dye (Orange G) incorporated in the agar at the start of electrophoresis to measure, by its visible rate of travel, the electrophoretic conditions being applied to some substance which itself is not visible. This eliminates careful measurements of voltage drop and/or current which need to be maintained if electrophoresis is to be described in electrical terms. The plastic matrix illustrated in Fig. 6 can be modified by having a $\frac{1}{16}$ inch hole drilled through it at one end just inside the point at which it will rest on the supporting tape. A 0.005 per cent solution of Orange G in buffer is placed in this hole, like antigen is placed in its holes, just before electrophoresis. Since the dye moves away from the cathode at a predictable rate under given electrophoretic conditions, one can assume that the same degree of electrophoresis has occurred in 2 separate runs if, in each, the Orange G indicator has moved the same distance.

As with the nonelectrophoretic micro diffusion tests in which plastic matrixes are used, agar under the matrix must not dry enough to draw away from the plastic. Hence, the trough and holes should be covered during electrophoresis, and during subsequent development of specific precipitates the slide should be kept in a humid atmosphere (e.g., in a Petri dish containing a moistened filter paper), and preferably in a refrigerator. In about 24 hours, reactions will be complete. Then, the matrix is pried off and the slide is washed, stained, and dried in the usual manner.

SUMMARY

Four basic modifications of the Wadsworth type of micro agar diffusion precipitin test are described to illustrate its various uses as an analytic tool for clinical and biologic laboratories.

The first, a single-diffusion technique, can be used to determine values similar to the classic optimal proportion ratio for one or more antigen-antibody systems in a single test. The second is a double diffusion quantitative technique particularly useful in the measurement of antigen or antiserum reactivity for several unequally reactive systems at one time.

The third modification is a very sensitive comparator technique with excellent resolution which has been devised in a way to maximize the chances of precipitin band convergence under adverse conditions such as obtain when a complex mixture of antigens which vary widely in concentration is being

compared with another similarly complex antigen mixture against a standard antiserum.

For the fourth modification, a Plexiglass matrix has been designed as the key piece of apparatus in a very simple but highly sensitive microimmunoelectrophoretic technique particularly adaptable to routine use.

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QUANTITATION OF GAMMA GLOBULINS IN HUMAN SERUM BY IMMUNOPRECIPITATION

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PRECISE determination of the concentration of gamma globulins in human serum has been of increasing importance since Bruton[1] described agammaglobulinemia in 1952. Subsequent case studies indicate that all degrees of hypogammaglobulinemia exist. Accurate diagnosis and treatment of individuals who are severely deficient in production of gamma globulins are greatly facilitated by a laboratory method capable of accurately quantitating gamma globulins in the range of 0 to 500 mg. per 100 ml. of serum.

Electrophoretic methods have in the past often been accepted as the standard of reference for quantitation of gamma globulins. The analysis of free or paper electrophoretic patterns provides satisfactory accuracy if the serum contains normal or increased concentrations of gamma globulins. However, the precision of these methods diminishes progressively with lower concentrations of gamma globulins. Sera which contain less than 100 mg. per 100 ml. cannot be quantitated by electrophoretic techniques with any significant degree of reliability.

Other established methods, such as measurement of gamma globulins after salting out with ammonium sulfate[2] or determination of turbidity with zinc sulfate,[3] are less precise than electrophoresis for measuring very low concentrations of gamma globulins. However, these techniques are useful for screening large numbers of sera to identify hypogammaglobulinemic specimens which require more accurate quantitation.

A number of laboratories have recently turned to various immunologic methods for the study of hypogammaglobulinemic sera. The erythrocyte agglutination inhibition method, described by Wiener,[4, 5] has been used to detect as little as 0.1 μg of gamma globulin,[6] but this method has not to our knowledge been adapted for precise quantitation. Recently, a latex fixation method,[7, 8] employing rabbit sera which contains antibodies to human gamma globulins, has been developed. This holds promise of being the simplest reliable screening test for the detection of severe hypogammaglobulinemia, but it does not seem at present that this method will be easily adaptable for accurate quantitation of concentration of gamma globulins.

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Immunoprecipitation is an established immunologic method which has recently been adapted[9-11] for quantitation of gamma globulins. This method is based upon the development of an animal serum which contains antibodies which will form a precipitate with human gamma globulins but will not precipitate with other serum proteins. The precipitate is quantitated by one of the standard protein methods and the value obtained is converted to milligrams of gamma globulins by means of a calibration curve. It is the purpose of this article to describe the development and use of these antisera in the study of hypogammaglobulinemic sera by immunoprecipitation.

METHODS

Preparation of Antigen.—Human gamma globulins prepared from pooled fresh serum as Cohn Fraction II by Methods 6 and 9[12] were used to inject laboratory animals. Gamma globulins from commercial sources appeared to be as satisfactory as those provided by Dr. J. L. Oncley or prepared in our own laboratory. Further refinement of these preparations of gamma globulins by starch zone electrophoresis did not seem to increase the likelihood of producing a rabbit antiserum free from contaminant antibodies which would precipitate with serum components other than the gamma globulins.

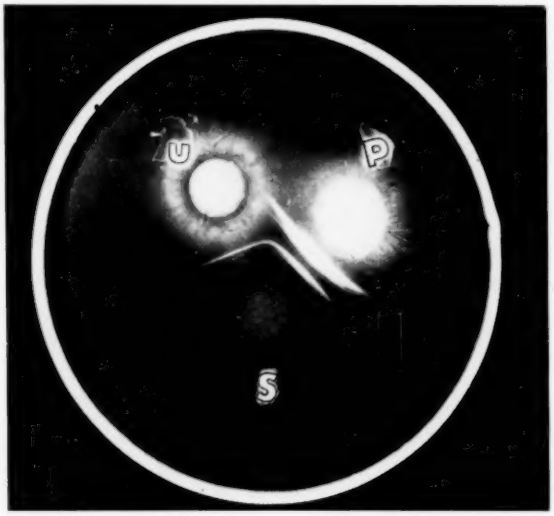

Fig. 1.—Ouchterlony plate illustrating the presence of contaminant antibodies. Well *S* contains whole human serum. Well *U* contains a pure or monospecific antiserum to gamma globulin. Well *P* contains a polyspecific serum which was deliberately made to contain many contaminant antibodies. The large multiple arcs immediately adjacent to *P* and the small arc adjacent to *S* were formed by contaminant antibodies. The fine continuous line with the sharp bend at its mid-point is that formed by the reaction of gamma globulins with anti-gamma globulins.

The antigen was modified for intravenous injection by alum precipitation[13] or for subcutaneous use by the addition of Freund's adjuvant.[14] It was observed that either route of administration resulted in high titers of antibody in rabbits.

Preparation of Antiserum.—Mature white rabbits weighing over 4 kilograms were reliable producers of precipitating anitbodies. They were immunized during a 4 week period by injection of a total dose of 7.5 to 10 mg. of protein per kilogram. If satisfactory titer of antibody developed, the animals were exsanguinated by cardiac puncture 1 week after the

last injection. The sera were pooled and heated to 56° C. for 30 minutes to destroy complement. Merthiolate was added as a preservative to a final concentration of 0.01 per cent.

An attempt was made to produce large single batches of antiserum by giving prolonged series of injections to 2 goats and 2 horses. Antisera produced in goats were unsatisfactory because the goats developed low titers of antibodies to gamma globulins and significant titers of contaminant antibodies. The horses developed blocking antibodies to gamma globulins, but did not form the desired precipitating antibodies.

Tests of Purity of Antiserum.—Four immunologic tests were applied to each batch of antiserum to determine whether it would precipitate serum components other than gamma globulins. Three of these tests were performed by reacting the rabbit antiserum with whole human serum in agar and noting the number of lines of precipitate which developed. The fourth test was performed by reacting fixed amounts of antiserum with increasing amounts of hypogammaglobulinemic human serum and plotting a curve of precipitate formed versus the amount of hypogammaglobulinemic serum in each tube.

Ouchterlony plates[15]*:* Agar was poured into Petri dishes in such a way that 3 wells were left. The wells were filled with the antiserum which was being tested, with a pure antiserum to gamma globulins, and with normal human serum, respectively. If more than one line of precipitate formed between the antiserum which was being tested and the human serum, it was assumed that the antiserum contained a contaminant antibody (Fig. 1). It was observed that this technique failed to detect low concentrations of contaminant antibodies which were detectable by other means.

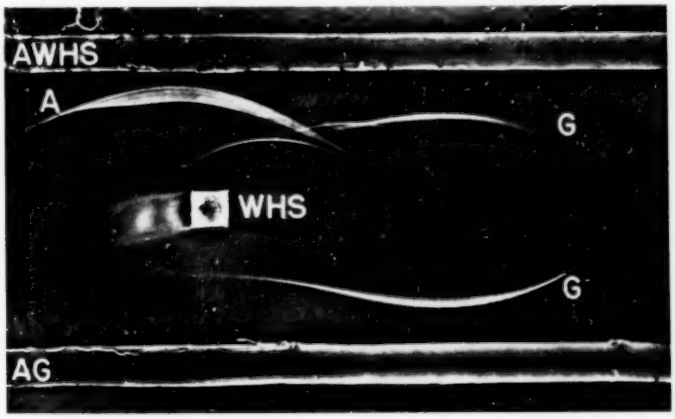

Fig. 2.—An immunoelectrophoretic plate illustrating the reaction between whole human serum (*WHS*) and 2 rabbit antisera. Serum *AWHS* contains antisera to major components of whole human serum and, therefore, forms multiple arcs of antigen-antibody precipitate; arc *A* is that from albumin and upper arc *G* is due to gamma globulins. The rabbit serum *AG* forms only the gamma globulin arc, which covers a wide electrophoretic spectrum.

Immunoelectrophoresis: Immunoelectrophoretic plates were made by the method of Grabar and Williams.[16] Human serum was placed in a square well near one end of a large rectangular agar plate, which was then subjected to electrophoresis so that the serum proteins were spread along the horizontal axis of the plate. The antisera which were being tested were then suspended in agar and poured into troughs cut near the long edges of the plate. As the antisera diffused toward the axis of the plate, antibodies reacted with their respective antigens and formed arcs of precipitate which could be partially identified by their positions (Fig. 2). This technique also failed to identify contaminant antibodies which were present in trace amounts, probably because the lines formed by these were too faint to be seen.

Oudin tubes[17]*:* Small bore, glass tubes were one half filled with an agar suspension

of the antiserum being tested. Whole human serum was poured on top of the agar and diffused downward through the suspended antiserum. Bands of precipitate formed and migrated downward through the agar at a rate roughly proportional to the concentration of the antigen.

In some instances, Oudin tubes proved sensitive enough to detect trace amounts of contaminant antibodies which formed a faint extra band in the agar. The usefulness of this method was limited by the fact that some antisera contained antibodies which formed lines in the Oudin tubes yet would not form a corresponding precipitate in a saline medium when reacted with the same human serum (Fig. 3). As an extreme example, 2 horse antisera were obtained which formed multiple distinct lines when reacted with human serum in an Oudin tube, but would form no precipitate when reacted with the same serum in saline.

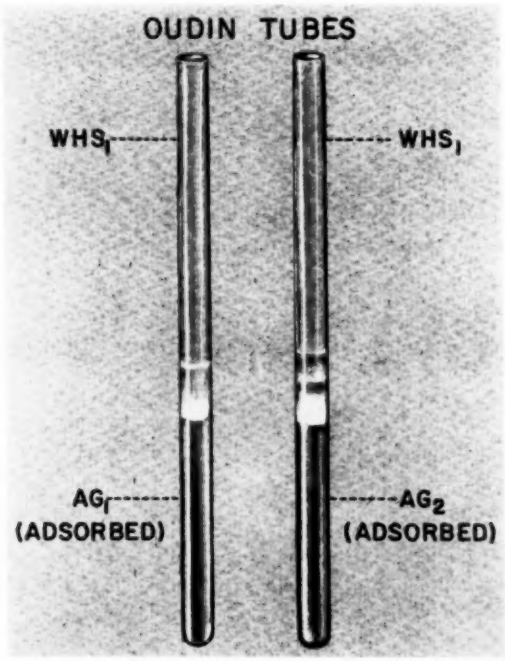

Fig. 3.—Oudin tubes illustrating the reaction of whole human serum *WHS*, against 2 different purified anti-gamma globulin sera, AG_1 and AG_2. The tube on the left shows one broad descending band of precipitate, as would be expected. The tube on the right has a definite second band even though other tests indicated that the antiserum in the lower half of the tube contained precipitating antibodies to gamma globulins only. It is possible that the upper band was formed by an "incomplete antibody" which precipitated with its antigen in agar but not in saline.

Detailed precipitation curves: Each batch of rabbit antiserum was further tested by reacting it with a hypogammaglobulinemic human serum in a saline medium, using the same procedure that was used in the final calibrations and quantitations. Fixed amounts of anti-serum were reacted in different tubes with many increments of hypogammaglobulinemic human serum, starting with very dilute solutions. The amount of precipitate formed was plotted against the amount of hypogammaglobulinemic serum added. When contaminant antibodies were present, these reacted with the normal protein groups of the hypogammaglobulinemic serum, forming a rounded peak near the origin of the curve (Fig. 4). It was necessary to use hypogammaglobulinemic serum for this test because normal human serum formed so much precipitate that the peak was obscured.

Removal of Contaminant Antibodies by Adsorption.—When cross-agglutinating antibodies were discovered, they were removed by the stepwise addition of hypogammaglobulinemic serum to the batch of antiserum. Centrifugation after each precipitation removed the undesired antibodies. There was no significant loss of anti-gamma globulins. The position of the peaks on the detailed precipitation curve indicated the minimum amount of hypogammaglobulinemic serum required. One milliliter of hypogammaglobulinemic serum usually provided adequate adsorption for 400 to 600 ml. of rabbit antiserum. Complete adsorption resulted in disappearance of the peak near the origin of the detailed precipitation curve determined with hypogammaglobulinemic serum (Fig. 4). After adsorption, the antisera were packaged in small vials and frozen.

Calibration of Antisera.—A calibration curve (Fig. 5) was obtained by reacting each antiserum with a solution of the same gamma globulins used to immunize the rabbits. Precipitations were performed in triplicate. The solution of gamma globulin was thoroughly

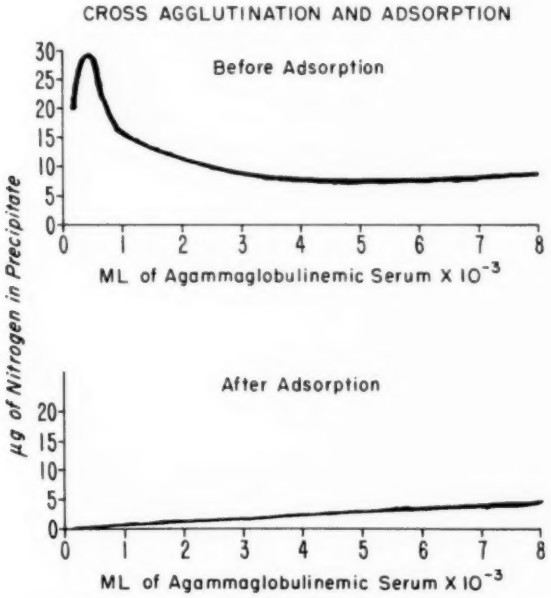

Fig. 4.—Precipitation curves illustrating the presence of contaminant antibodies which formed the peak near the origin of the upper curve. The lower curve illustrates that adsorption of these antibodies by the addition of agammaglobulinemic serum eliminated the contaminant antibodies.

mixed with either 0.2 or 0.4 ml. of the rabbit antiserum; the amount used depended upon the antibody titer. The tubes were allowed to stand for 1 hour at room temperature and 48 hours at 4° C.; longer storage did not significantly increase precipitation.

The tubes were then centrifuged at a cold temperature, the supernate was decanted, and cold saline was added as a wash. The tubes were jarred to break up the precipitate, which was then reassembled by centrifugation at a cold temperature. This was repeated. The precipitate was then quantitated by a modified Folin method.[18]

Quantitation of Serum Specimens.—Quantitations of gamma globulins in human sera were performed by the same method that was employed for the calibration. A dilution of the human serum specimen was chosen so that the amount of precipitate formed fell on the relatively straight early portion of the calibration curve. The quantity of gamma globulin present in the serum specimen could then be determined from the calibration curve.

Electrophoretic Determinations.—The normal human sera were quantitated by paper electrophoresis for comparison with the values obtained by immunoprecipitation. The electrophoretic analyses were performed according to the Spinco procedure,* with the exception that the ionic strength of the Veronal buffer used was 0.10M, which necessitated a running time of 19 hours with a current output of 8 Ma. for 1 cell. After staining with bromphenol blue, quantitations of the major serum components were made by means of a Spinco Analytrol.

RESULTS

The reproducibility of the immunoprecipitative quantitation was determined to be excellent. In a consecutive series of 251 sets of triplicate analyses of normal and hypogammaglobulinemic sera, the coefficient of variation[23] was 5 or

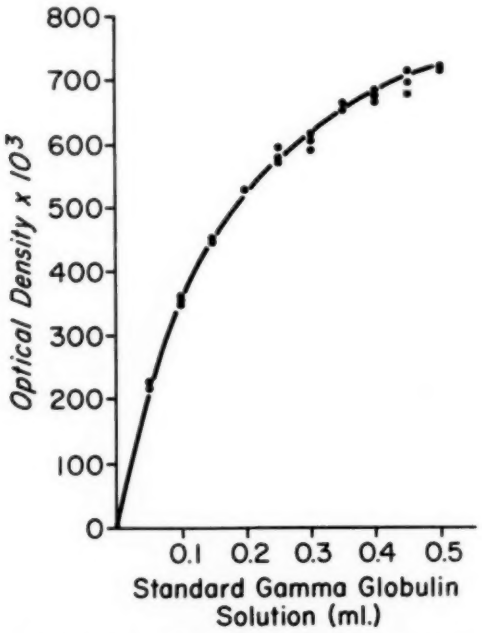

Fig. 5.—A typical calibration curve determined by reacting a fixed amount of antiserum with increasing increments of a standard solution of gamma globulins. The portion of the curve from 200 to 400 O.D. × 10^3 provided the most reliable quantitations. Readings are always taken in such zones of antibody excess.

less in 90 per cent of the determinations and 2 or less in 53 per cent. The reproducibility obtainable in the hypogammaglobulinemic range is illustrated in Table I by a tabulation of quintuplicate determinations upon representative sera from various degrees of hypogammaglobulinemia. These and other data indicate that quantitative immunoprecipitation by the technique herein described may provide reproducible results in the low concentrations of gamma globulins, where accuracy is very helpful for the precise diagnosis and treatment of hypogammaglobulinemic syndromes.

By this method, a range of 945 to 1,911 (mean 1,403) mg. per cent of gamma globulins was found in 28 sera of normal adults 20 to 40 years of age. Differen

*Operating Instructions: Spinco Model R-Series C-Paper Electrophoresis Apparatus, Pamphlet 16R-1054, edited by Special Instruments Corporation, Belmont, Calif.

batches of rabbit antiserum yielded slightly different results, as noted in Table II. Therefore, it seemed important to check each separate pool of antiserum against reference specimens of normal sera to determine the degree of variation between pools.

This immunoprecipitation technique consistently gave a higher value for the concentration of gamma globulins in a given serum than did paper electrophoresis. The degree of this difference encountered in analyzing 28 normal sera is depicted in Table II and in Fig. 6.

TABLE I. COMPARISON OF DAILY DETERMINATIONS, HYPOGAMMAGLOBULINEMIC SERA

PATIENT	MG. %	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION
S-H.	168			
	150			
	165	161	8.8	5.51
	170			
	150			
R.	189			
	192			
	198	202	11.2	5.57
	213			
	217			
P.	411			
	425			
	440	424	9.6	2.27
	425			
	418			
H.	514			
	498			
	499	532	37.8	7.12
	556			
	593			

Each daily determination listed is the average of 6 individual quantitations; therefore, the standard deviation and coefficients of variation for each serum sample are based upon 30 precipitations using 1 rabbit antiserum.

The accuracy of immunoprecipitation in the hypogammaglobulinemic range was satisfactory, and it is this characteristic which makes the method valuable. Fig. 7 presents the results obtained when sera from 9 patients with various degrees of hypogammaglobulinemia were quantitated with 4 different batches of rabbit antiserum. Of particular note are the 2 sera which contain the lowest concentrations of gamma globulins, both from boys with congenital "agammaglobulinemia." The values obtained ranged from 0.6 to 1.0 mg. per 100 ml. for one of these patients and from 3.8 to 5.3 mg. per 100 ml. for the other. These are in a range which cannot be quantitated by other methods.

DISCUSSION

The gamma globulins can be defined only in general terms, and there is, therefore, no absolute standard against which any quantitative method can be evaluated. The electrophoretic definition implies that any serum protein which migrates within a given range of mobilities (-0.97×10^{-5} to -2.63×10^{-5} cm.^{2} per volt per second when the buffer is diethylbarbituric acid and sodium di-

ethylbarbiturate, pH 8.6, and ionic strength 0.1)[21] is gamma globulin regardless of its other characteristics. Studies such as immunoelectrophoresis[16] (Fig. 2) and combined electrophoresis and ultracentrifugation[22] suggest that there are "gamma globulins" which migrate electrophoretically as rapidly as alpha$_2$

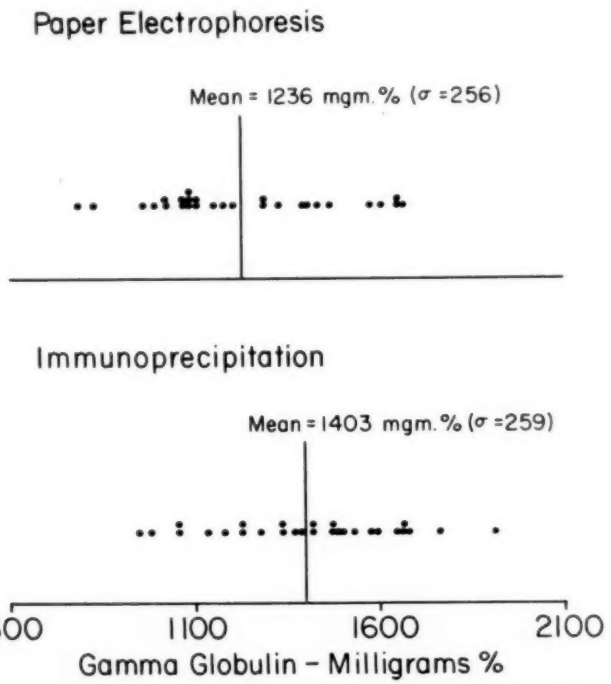

Fig. 6.—When normal sera were quantitated by paper electrophoresis, the values obtained were consistently lower than when the same sera were quantitated by immunoprecipitation. The lower scattergram indicates the average values obtained when 28 sera were quantitated with 4 rabbit antisera. The upper scattergram indicates the results obtained when the same sera were quantitated by paper electrophoresis.

globulins but which have immunologic or sedimentation characteristics identical to gamma globulins. It seems reasonable to assume that these rapidly migrating portions of the gamma globulins, which are not included in electrophoretic quantitations, account for our observation that immunoprecipitation in a given serum may give a higher value for concentration of gamma globulins than does paper electrophoresis. The immunoprecipitation method defines the gamma globulins as those proteins which may be precipitated from human serum by an antiserum prepared by injecting animals with gamma globulins. This definition implies that an antigenic portion of the molecule rather than its mobility in an electrophoretic field determines whether it should be termed a gamma globulin.

Immunologic methods are well established for the detection of trace amounts of proteins and for determinations of titers of antibodies or of antigens. When immunologic methods are used quantitatively, inaccuracies may be introduced because of the complex nature of the antibody-antigen reaction. One major problem, that of contaminant, or cross-agglutinating antibodies, has been discussed above.

There are several other potential sources of error which are difficult to evaluate by existing methods. For instance, different portions of the gamma globulins might combine with antibody in different ratios. Also, the standard solution used to calibrate the antisera might contain protein which was antigenically inactive. If these factors had been important, it is likely that inconsistencies would have been observed when a given serum was quantitated with several different antisera.

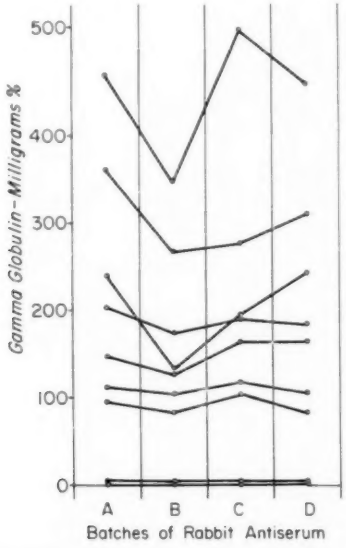

Fig. 7.—Results obtained when 9 hypogammaglobulinemic sera were quantitated using the same 4 batches of rabbit antiserum illustrated in Fig. 6. Each point represents the average of 2 sets of triplicate determinations.

The normal range for gamma globulins of adults as determined by free electrophoresis is quite wide. For instance, Rafsky and associates[19] determined that the sera of 31 aged normal men contained 1,200 ± 200 mg. (S.D.) of gamma globulin per 100 ml. Their over-all range was from 600 to 1,600 mg. per 100 ml. Reiner and associates[20] reported that the values obtained by free electrophoretic analysis of the sera from 80 blood donors was 1,000 ± 210 mg. (S.D.) per 100 ml.

TABLE II. QUANTITATION OF GAMMA GLOBULINS IN 28 NORMAL SERA

METHOD	NUMBER OF SERA	CONCENTRATION, MG. %	
		MEAN	RANGE
Immunoprecipitation rabbit antiserum pool			
A	28	1,478	994-1,926
B	28	1,320	897-1,817
C	22	1,516	944-2,011
D	16	1,392	969-1,890
Paper electrophoresis	28	1,236	792-1,666

High, low, and mean values for gamma globulins determined by quantitation of sera from normal adults who ranged in age from 20 to 40 years; 4 different batches of rabbit antiserum and paper electrophoretic strips quantitated by means of a Spinco Analytrol scanner were used. The rabbit antisera were prepared with the following antigens: Batch A and B, gamma globulins prepared in University of Washington laboratory, injected intravenously; Batch C, gamma globulins prepared by Cutter Laboratories, injected intravenously; and Batch D, gamma globulins prepared by Cutter Laboratories, injected subcutaneously.

Two laboratories in which immunoprecipitation is used have reported small groups of normal values. Goodman and associates,[11] using a chicken antiserum, determined a gamma globulin concentration of 1,200 ± 180 mg. per 100 ml. in 45 normal adult subjects; a close agreement with results by paper electrophoresis was reported. Zak and Good[10] reported a mean value of 1,124.3 mg. per 100 ml. with a range from 734.0 to 1,687.5 for the sera of 24 young normal adults using a rabbit antiserum for immunoprecipitation.

SUMMARY

1. Immunoprecipitation is a useful and precise method for measuring the concentration of gamma globulins in human sera. Its principal value is in the study of hypogammaglobulinemic sera, for which precise measurement of very small quantities of gamma globulins is of importance.

2. Other methods of quantitating gamma globulins, such as salting out, turbidity, and electrophoretic techniques, are incapable of the precise measurement of low concentrations of gamma globulins which can be obtained by immunologic methods.

3. Essential to the immunoprecipitation method is the availability of an antiserum capable of precipitating with all the gamma globulins but not with other serum components. Agar techniques may fail to indicate correctly the presence or absence of undesired contaminant antibodies which will precipitate serum components other than gamma globulins.

4. Contaminant antibodies can be detected by the study of the precipitation curve obtained when antiserum is reacted with high dilutions of hypogammaglobulinemic serum. These antibodies can be removed by adsorption with hypogammaglobulinemic serum.

5. Each batch of antiserum must be calibrated by means of a standard solution of gamma globulin and the range of normal values must be evaluated with sera of known concentration of gamma globulins.

6. Concentrations of gamma globulins obtained when normal sera were quantitated by immunoprecipitation tended to be higher than corresponding values obtained when the same sera were analyzed by electrophoretic methods. Probably this difference was due to the presence of proteins which reacted immunologically as gamma globulins but which, during electrophoresis, migrated more rapidly than the bulk of gamma globulins.

7. Immunoprecipitation was capable of measuring levels of gamma globulins as low as 1 to 5 mg. per 100 ml. with excellent reproducibility. When hypogammaglobulinemic sera were quantitated with the use of a single batch of antiserum, the coefficients of variation ranged from 5 to 7. There is no absolute standard against which these determinations could be compared, but there can be little doubt that they represented a true measure of the immunologically active gamma globulins present.

We are grateful to Drs. John L. Oncley of Boston, Mass., and John H. Hink, Jr., of the Cutter Laboratories of Berkeley, Calif., for generous supplies of gamma globulins, and to Dr. J. H. Brown of Wyeth Laboratories, Inc., Marietta, Pa., for attempting to produce

suitable anti-gamma globulin serum in 2 horses. Sera from agammaglobulinemic patients employed for adsorption of rabbit antisera were kindly furnished by Drs. Robert A. Good of Minneapolis, Minn., and Christopher M. Martin of Bethesda, Md. Dr. Patrick D. Goldsworthy assisted in the fractionation and physicochemical analysis of gamma globulins, Dr. Russell S. Weiser advised concerning immunologic methods and interpretation of the results, and Dr. Blair M. Bennett supervised the statistical treatment of final data.

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THE COLORIMETRIC DETERMINATION OF ARGININE ESTER HYDROLYSIS BY HUMAN SERA

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RECENT reports have renewed interest in the possible presence of proteolytic and related enzymes of pancreatic origin in the human serum. These reports show that, in acute and chronic pancreatitis and in carcinoma of the pancreas, activities attributed to trypsin,[1] active tryptic enzymes,[2] arginine exopeptidase,[3] and leucine aminopeptidase[4] are increased. However, the presence of trypsin in human plasma and the origin of the other so-called pancreatic peptidases remains unsettled.[5] The hydrolysis of arginyl esters provides a quantitative test for several enzymatically active proteins.[6-12] Three of these proteins, plasmin (fibrinolysis), thrombin, and the first component of complement, are present in the human plasma. Human serum which contains streptokinase shows an increased rate of arginyl ester hydrolysis which equals the rate of activated plasminogen.[13]

The enzymatic hydrolysis of α-benzoyl-L-arginine ethyl ester and α-benzoyl-L-arginine amide may be followed by the methods of Schwert, et al.[14] or Iselin and Niemann[15] which determine either the free carboxyl groups or ammonia liberated. In the present study a sensitive colorimetric means of detecting organic esters was further investigated and was applied to the measurement of serum hydrolysis of α-benzoyl-L-arginine ethyl ester, p-toluene-sulfonyl-L-arginine methyl ester, and L-lysine methyl ester. The colorimetric means of detecting organic esters was introduced by Feigl[16] as a spot test and later as a quantitative test by Hestrin[17] and Fruton.[18] The reactions for the measurement of enzymatic activity are formulated in Fig. 1. The test involves the incubation of a measured amount of buffered substrate with serum and the determination of the amount of the ester which remains after incubation. The results are expressed in units of activity which equal the number of micromoles of α-benzoyl-L-arginine ethyl ester hydrolyzed per hour per milliliter of serum.

METHOD

Reagents.—

a. The 0.02M substrate was prepared by dissolving 0.3428 Gm. of α-benzoyl-L-arginine ethyl ester hydrochloride† in distilled water to a volume of 50 ml. This substrate had a pH of approximately 3.6, and was stable for several weeks at room temperature. At a pH o

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†The α-benzoyl-L-arginine ethyl ester hydrochloride, Lot No. A 4603, was obtained chromatographically pure from Mann Research Laboratories, 136 Liberty Street, New Yor 6, N. Y.

7.8, a 20 mcg. per milliliter sample underwent spontaneous hydrolysis (about 0.2 mcg. per hour at 37° C.). The rate of spontaneous hydrolysis increased as the pH was raised; at a pH of 11 about 70 per cent of the arginyl ester was hydrolyzed per hour.

b. The 0.10M Veronal buffer (pH 7.8) was prepared according to the method of Michaelis.[19] Ten and three tenths grams of sodium diethylbarbiturate (Veronal sodium) was dissolved in distilled water to a volume of 500 ml. To 66.2 ml. of this solution was mixed 33.8 ml. of 0.10M HCl, and the 0.10M sodium Veronal buffer solution with a pH of 7.8 was obtained.

c. Alkaline-hydroxylamine solution: 13.9 per cent (2M) hydroxylamine hydrochloride (Eastman No. 340) was nearly neutralized with an equal volume of 14 per cent (3.5M) sodium hydroxide. The alkaline-hydroxylamine solution was prepared just prior to use and was good for at least 3 hours. The stock hydroxylamine hydrochloride was stored at 4° C. when not in use.

d. Approximately 3 normal hydrochloric acid: One volume of concentrated HCl was diluted with 3 volumes of distilled water.

e. Ten per cent ferric chloride solution: 10 per cent (0.37M) ferric chloride ($FeCl_3.6H_2O$)* was prepared in 0.10N HCl.

f. Ten per cent trichloroacetic acid.

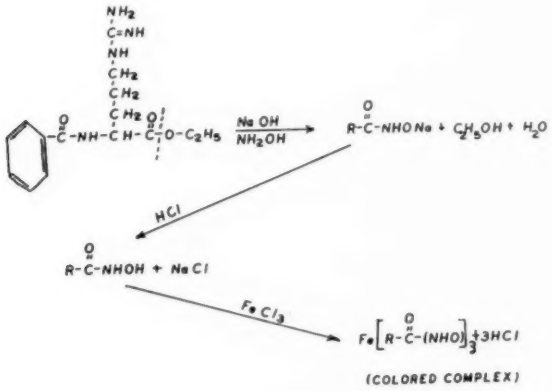

Fig. 1.—The reactions employed for the determination of α-benzoyl-L-arginine ethyl ester hydrochloride. The dotted line indicates the position which is attacked by the serum enzymes.

Procedure.—

1. One milliliter of substrate (containing 20 μM of α-benzoyl-L-arginine ethyl ester) and 1 ml. of Veronal buffer were added to each of 2 test tubes (16 by 100 mm.), A and B. Both tubes were placed in the incubator at 37° C. for 10 minutes.

2. Five-tenths milliliter of serum was then added to Tube A, was mixed, and was incubated for exactly 1 hour.

3. Both tubes were removed from the water bath and 1.5 ml. of 10 per cent TCA was added to Tube A.

4. Five-tenths milliliter of serum was added to Tube B, followed by 1.5 ml. of 10 per cent TCA.

5. The contents in each tube were thoroughly mixed, allowed to stand for 5 minutes, and were centrifuged at approximately 2,500 r.p.m. for 10 minutes.

*Merck & Co., Inc., reagent grade No. 72741.

6. Two milliliters of clear supernatant fluid was withdrawn from each tube and placed in Coleman cuvettes.

7. To cuvettes A, B, and C was added 2 ml. of alkaline-hydroxylamine solution; after thorough mixing the cuvettes were allowed to stand for at least 1 minute. Cuvette C (blank) contained 2 ml. distilled water.

8. One milliliter of dilute hydrochloric acid was added and mixed.

9. Finally, 2 ml. ferric chloride solution was added and mixed, and the cuvettes were allowed to stand for exactly 15 minutes.

10. The readings were made in a Coleman spectrophotometer (Model 14), at 540 mμ with the blank Tube C set at zero optical density.

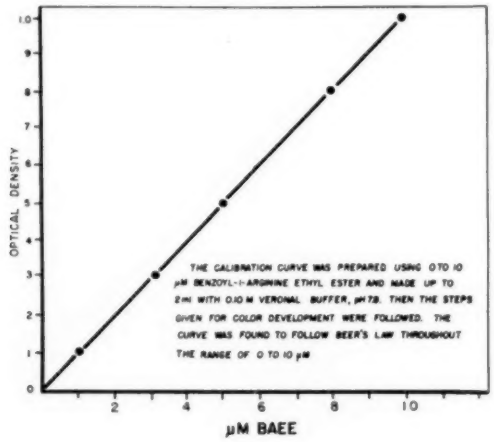
OPTICAL DENSITY
THE CALIBRATION CURVE WAS PREPARED USING 0 TO 10 μM BENZOYL-L-ARGININE ETHYL ESTER AND MADE UP TO 2ml WITH 0.10 M VERONAL BUFFER, pH 7.8. THEN THE STEPS GIVEN FOR COLOR DEVELOPMENT WERE FOLLOWED. THE CURVE WAS FOUND TO FOLLOW BEER'S LAW THROUGHOUT THE RANGE OF 0 TO 10 μM
μM BAEE

Fig. 2.

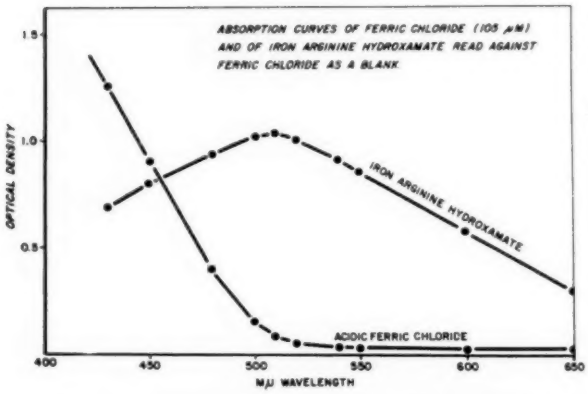
ABSORPTION CURVES OF FERRIC CHLORIDE (105 μM) AND OF IRON ARGININE HYDROXAMATE READ AGAINST FERRIC CHLORIDE AS A BLANK
OPTICAL DENSITY
IRON ARGININE HYDROXAMATE
ACIDIC FERRIC CHLORIDE
mμ WAVELENGTH

Fig. 3.—The acidic ferric chloride (blank) was prepared using 2 ml. of 0.05M barbital buffer. The iron arginine hydroxamate solution was prepared using 8 μM of α-benzoyl-L-arginine ethyl ester *(BAEE)* in 2 ml. of barbital buffer. Both were treated according to the procedure described under color development. The acidic ferric chloride solution was read against distilled water. The iron arginine hydroxamate was read against the acidic ferric chloride as a blank.

Calibration Curve.—The calibration curve (Fig. 2) was prepared from readings obtained with using 0 to 10 μM of buffered α-benzoyl-L-arginine ethyl ester. The loss of

α-benzoyl-L-arginine ethyl ester due to spontaneous hydrolysis and coprecipitation with serum proteins was less than 1 μM.

The reaction between alkaline-hydroxylamine and α-benzoyl-L-arginine ethyl ester or L-lysine methyl ester was complete within 2 minutes, whereas the reaction with p-toluene-sulfonyl-L-arginine methyl ester required 30 minutes for completion. When 10 μM of each of the above esters per 2 ml. of Veronal buffer were treated according to the color development procedure they were found to have optical densities of 1.30 (p-toluene-sulfonyl-L-arginine methyl ester), 1.00 (α-benzoyl-L-arginine ethyl ester), and 0.66 (L-lysine methyl ester).

A convenient stable reference standard was found to be succinylhydroxamate. This stock hydroxamate was prepared by dissolving 1.007 Gm. of succinic anhydride (Eastman No. 868) in 50 ml. of the alkaline-hydroxylamine solution and diluting to a volume of 100 ml. with distilled water. The working reference standard (5 μM per milliliter) was prepared by diluting to a volume of 100 ml. with distilled water. When 5 μM of the succinylhydroxamate per 2 ml. of buffered Veronal was treated according to the color development procedure, the optical density was 1.06.

The maximum color intensity of the arginine iron hydroxamate developed when the final Fe^{+++} concentration was over 100 μM per milliliter. When the concentration of the Fe^{+++} was increased the optical density was not increased when read against its blank of acid ferric chloride. When iron-binding buffers such as 0.1M phosphate were used, the maximum color intensity was obtained when the Fe^{+++} concentration was 200 μM per milliliter. At the concentration of Fe^{+++} (105 μM per milliliter) used in the method presented and reading at the wavelength of 540 mμ, the blank was equivalent to less than 0.5 μM of α-benzoyl-L-arginine ethyl ester. Reducing the Fe^{+++} concentration to 50 μM per milliliter produced a more violet color which was not stable, and only 52 per cent of the maximum color development. The optimal stability of the iron complex was obtained between 10 and 20 minutes.

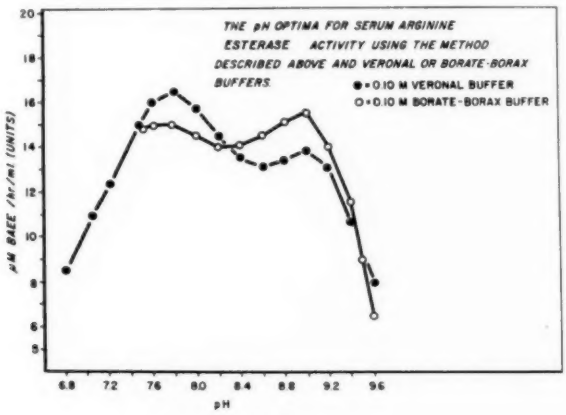

Fig. 4.

As shown in Fig. 3, the maximum absorption of the iron complex was obtained at wavelength of 510 mμ, when read against the acidic ferric chloride blank. An absorption curve of the acidic ferric chloride blank read against distilled water indicates that the minimum interference caused by the ferric chloride occurs at those wavelengths over 530 mμ.

Measurement of Enzyme Activity.—One unit was considered as equivalent to 1 μM of benzoyl-L-arginine ethyl ester hydrolyzed per hour per milliliter of serum.

The calculations are as follows: the colorimetric of Tubes A and B are converted into micromoles by referring to the calibration curve. Since the micromoles represented in the

aliquots tested are one-half of the total present, the micromoles read from the chart are multiplied by 2. The total micromoles present in Tube A substrated from the total present in Tube B are equal to the number of micromoles of α-benzoyl-L-arginine ethyl ester hydrolyzed per hour per 0.5 ml. of serum. Multiplication of this value by 2 gives the number of micromoles per hour per milliliter. The calculations may be simplified as follows: convert the optical densities of Tubes A and B into micromoles of α-benzoyl-L-arginine ethyl ester; then (micromoles in Tube B − micromoles in Tube A) × 4 units (micromoles α-benzoyl-L-arginine ethyl ester hydrolyzed per hour per milliliter).

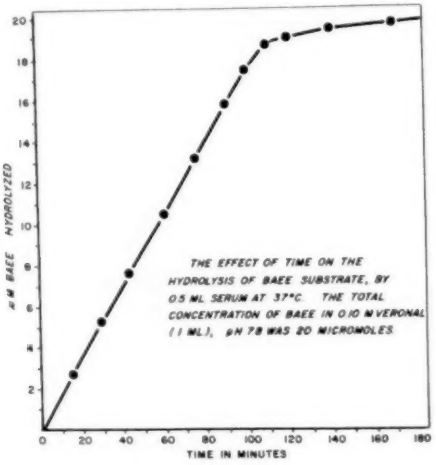
μM BAEE HYDROLYZED
THE EFFECT OF TIME ON THE HYDROLYSIS OF BAEE SUBSTRATE, BY 0.5 ML SERUM AT 37°C. THE TOTAL CONCENTRATION OF BAEE IN 0.10 M VERONAL (1 ML), pH 7.8 WAS 20 MICROMOLES.
TIME IN MINUTES

Fig. 5.

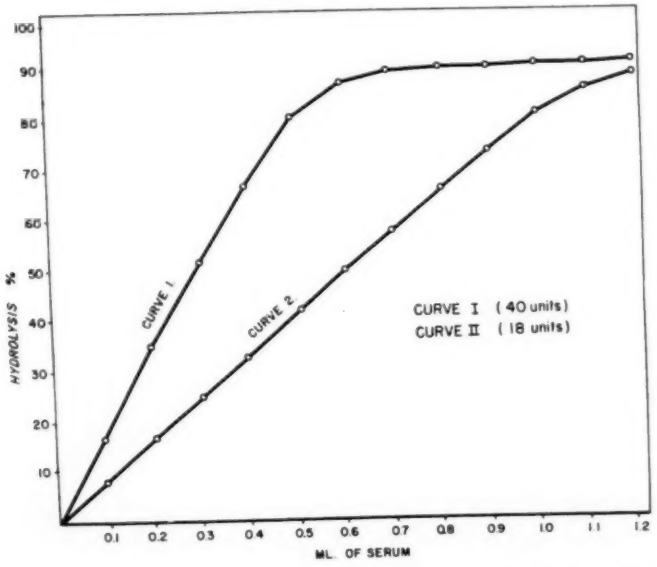

Fig. 6.—The effect of serum concentration on the per cent of *BAEE* hydrolysis. Two sera, one from a normal male donor (18 units), the other from a patient with chronic pancreatitis (40 units), were used. Twenty micromoles of *BAEE* in 0.10 Veronal, pH 7.8, with varying amounts of sera was incubated for 1 hour at 37° C.

Blanks of similar amounts of sera were used to correct for any loss of substrate caused by coprecipitation with the serum proteins.

Enzyme Kinetic Considerations.—The effect of pH, time, concentrations of serum enzymes and substrate, inhibitors, and accelerators were determined in order to obtain the optimal conditions for the enzymatic hydrolysis of arginyl esters.

Effect of pH on the hydrolysis of α-benzoyl-L-arginine ethyl ester. The optimal pH values for the hydrolysis of the ester in 0.10M Veronal buffer were 7.8 and 9.0, as shown in Fig. 4. Higher molar concentrations of Veronal were not used because of the tendency of barbituric acid to precipitate. Lower concentrations of Veronal did not effectively buffer the amount of serum used (0.5 ml.) as well as did the 0.10M buffer. The 0.10M borate buffers showed optimal pH values and blanks similar to those obtained with Veronal. The 0.10M Tris buffers showed an optimal pH value of 8.0.

Effect of time on the hydrolysis of α-benzoyl-L-arginine ethyl ester by serum is shown in Fig. 5. The rate of hydrolysis which employed serum of 21 units was linear up to 90 minutes. Incubation beyond this time and up to 180 minutes did not result in the hydrolysis of all of the substrate present. The decline in the rate of hydrolysis was not due to thermal inactivation, since serum preincubated at 37° C. for 1 hour, and then incubated with the substrate exhibited a similar die-away curve.

Effect of serum (enzyme) concentration on the hydrolysis of α-benzoyl-L-arginine ethyl ester is shown in Fig. 6. Two sera were used in this experiment, one from a patient with acute pancreatitis, with an activity of 40 units (*Curve I*), and the other from a normal donor with an activity of 18 units (*Curve II*). In both cases the per cent of hydrolysis varied directly as the concentration of the sera, and hydrolysis of the substrate proceeded according to zero order kinetics until approximately 80 per cent of the substrate was hydrolyzed. At this point a marked decrease in the per cent of hydrolysis was noted. After 88 per cent of the substrate was hydrolyzed the additions of increasing amounts of serum did not significantly increase the per cent of hydrolysis.

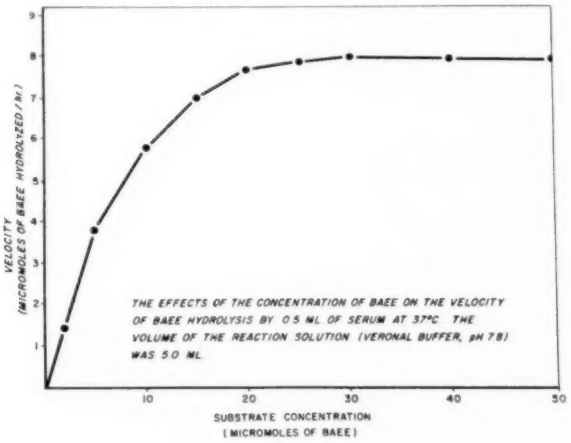
VELOCITY
(MICROMOLES OF BAEE HYDROLYZED / hr.)
THE EFFECTS OF THE CONCENTRATION OF BAEE ON THE VELOCITY OF BAEE HYDROLYSIS BY 0.5 ML OF SERUM AT 37°C. THE VOLUME OF THE REACTION SOLUTION (VERONAL BUFFER, pH 7.8) WAS 5.0 ML.
SUBSTRATE CONCENTRATION
(MICROMOLES OF BAEE)

Fig. 7.

Effect of substrate concentration is shown in Fig. 7. As the substrate concentration was increased up to 20 μM, the rate of hydrolysis also increased. Beyond 20 μM concentration of α-benzoyl-L-arginine ethyl ester, the velocity of arginyl ester hydrolysis remained at the maximum rate of 8 μM per hour.

Effects of the end products (α-benzoyl-L-arginine and ethanol) on the hydrolysis of α-benzoyl-L-arginine ethyl ester by serum were studied. Amounts of α-benzoyl-L-arginine hydrochloride from 0 to 100 μM were adjusted to a pH of approximately 7.8 and incorporated into the 20 μM per milliliter of buffered arginyl ester substrate. The samples were then incubated and treated as described above. The results indicated that these concentrations of

end products did not change the mount of arginyl ester hydrolyzed. Concentrations of α-benzoyl-L-arginine greater than 120 μM per milliliter of substrate caused a detectable decrease in the amount of arginyl ester hydrolyzed.

When the amount of ethanol incorporated in the substrate was increased from 1 to 10 per cent, a concomitant increase in the amount of substrate hydrolyzed was noted. Concentrations beyond 10 per cent and up to 50 per cent ethanol caused a steady reduction in the per cent of substrate hydrolyzed. It was also noted that increasing concentrations of ethanol beyond 20 per cent caused an increase in turbidity or protein precipitation.

The effects of several known accelerators and inhibitors of plasmin, trypsin, cathepsin, or esterase were studied. Several α-benzoyl-L-arginine ethyl ester substrates were made to contain either 25 μM NaF, 50 μM cysteine, 50 μM ascorbic acid, or 1 mcg. pepsin* per milliliter. The percentage of arginyl ester hydrolyzed was not affected by these compounds. The addition of copper sulfate to the buffered substrate caused complete inhibition of the arginyl ester hydrolysis when the Cu^{++} concentration was 10 μM per milliliter. The addition of 0.2 to 0.5 mg. of ovomucoid or soybean inhibitor resulted in a 28 per cent reduction in the hydrolysis of the arginyl ester by serum. Complete inhibition was not effected until 20 mg. of ovomucoid was added.

The effects of purified trypsin added to serum were studied. Purified trypsin† (0.16 mg. protein N per milliliter) was prepared in 10^{-2}M $CaCl_2$ and the pH was adjusted to 3 with diluted hydrochloric acid. The trypsin solution was diluted 1:200 with distilled water and 0.1 ml. was incubated for 1 hour with 20 μM of arginyl ester in Veronal buffer containing 0.002M $CaCl_2$. The rate of hydrolysis was 10 μM per hour at 37° C. Ovomucoid (0.2 mg.) or soybean inhibitor (0.2 mg.) completely inhibited the activity of the diluted trypsin. When the diluted trypsin (0.1 ml.) was added to 0.5 ml. of serum the percentage of arginyl ester hydrolyzed was 32 per cent greater than the added activities of the serum and trypsin. The addition of ovomucoid did not decrease the activity of the serum-trypsin combination by more than 15 per cent. When the ovomucoid was added to the trypsin prior to the serum addition, the activity was nearly equal to that of the serum alone.

The effects of streptokinase added to the serum were studied. To 0.1 ml. of purified streptokinase† (10,000 units) and 0.3 ml. of distilled water was added 0.1 ml. of serum. The procedure given above was then followed. Both highly purified streptokinase and the commercial streptokinase (Varidase) preparations were equally effective as activators for the serum hydrolysis of arginyl and lysyl esters. The addition of 1,000 units of streptokinase caused a tenfold increase in the amount of arginyl ester hydrolysis.

The hydrolysis of α-benzoyl-L-arginine ethyl ester by various whole blood components and urine was demonstrated. One milliliter of whole blood (4 samples) was washed 3 times with normal saline and was found to hydrolyze 1 to 3 μM of arginyl ester per hour. Normal sera (7 donors) hydrolyzed between 14 and 19 units. When the same sera were activated with streptokinase (10,000 units) the values were between 170 and 210 units. Oxalated plasma from the same donor subjects hydrolyzed between 10 and 14 units. Normal urine (2 subjects) dialyzed at 4° C. for 24 hours with several changes of distilled water and when lyophilized showed 5 and 7 μM of ester hydrolyzed per 100 ml. of urine.

The hydrolysis of several amino acid esters was investigated. Various amino acid ester substrates (20 μM per milliliter) were prepared in 0.1M Veronal (pH 8.0) or 0.1M acetate (pH 5.0) buffers and treated according to the above procedure. Benzoyl glycine methyl ester, glycine methyl ester, L-leucine ethyl ester, L-phenylalanine ethyl ester, benzoyl DL-methionine methyl ester, and L-tyrosine hydroxamide were not detectable when hydrolyzed at either pH 5.0 or 8.0. At a pH of 5.0 the following esters were hydrolyzed by serum: L-lysine methyl ester (3 units), L-tyrosine ethyl ester (0.6 unit), acetyl-L-tyrosine ethyl ester (3 units), and benzoyl-L-tyrosine ethyl ester (4 units). The clinical significance and pH optimum of acetyl-L-tyrosine ethyl ester hydrolysis is currently being investigated.

*The purified trypsin and pepsin were obtained twice recrystallized salt free preparations Nos. 629 and 627, respectively, from Worthington Biochemical Corp., Freehold, N. J.

†Supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y.

DISCUSSION

A classification and delineation of the serum enzymes responsible for the hydrolysis of arginyl esters have not been established. Studies on pH optima of arginyl ester hydrolysis clearly indicates the presence of at least two enzymes, one with a pH optimum of 7.8 and the other with a pH optimum of 9.0. The results presented here suggest that trypsin activates a serum proenzyme, a finding also noted by Sherry, et al.[20] The results here also suggest that human serum proteins probably interact with ovomucoid and render it ineffective as a trypsin inhibitor. The competitive inhibition of α-benzoyl-L-arginine on the serum hydrolysis of its ester demonstrated in this work has not been previously noted.

The rates of arginyl ester hydrolysis by plasma are 30 to 40 per cent lower than those obtained with the same subject's serum. This difference may be due to the presence of thrombin in human serum. Therefore, the serum was incubated for one hour at 37° C. to allow for thermal and antithrombin inactivation of the thrombin. The serum values were still higher than those obtained with plasma. Either the thrombin was not destroyed by this incubation treatment or there is present in the clotting mechanism an enzyme other than thrombin which hydrolyzes arginyl esters.

SUMMARY

A simple colorimetric method for the determination of the activity of human serum on α-benzoyl-L-arginine ethyl ester is described, and some data on the kinetic considerations of these enzymes are given. The number of micromoles of α-benzoyl-L-arginine ethyl ester hydrolyzed per hour per milliliter (units) obtained from 7 normal males was between 14 and 19. The normal total activity (streptokinase activated) appears to be between 170 and 210 units.

The author wishes to thank Dr. Philip M. LeCompte for his helpful suggestions in the preparation of this report.

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A NEW ASSAY FOR FACTOR V (PROACCELERIN-ACCELERIN) USING RUSSELL'S VIPER VENOM

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ALTHOUGH factor V has a definite role in both the intrinsic and the extrinsic blood clotting systems, all of the proposed assays for its activity employ the latter role because of the greater accuracy of assay methods for the extrinsic system. If a system contains optimal amounts of tissue thromboplastin, prothrombin, proconvertin, Stuart-Prower factor, calcium, and fibrinogen, its clotting time will, according to present knowledge, depend on the concentration of added factor V.

Two types of such systems have been proposed. One is based on the use of plasma from a patient congenitally deficient in factor V, but normal with respect to other clotting factors. The other is based on the use of normal plasma which has been treated in one of a number of ways designed to artificially reduce factor V without significantly affecting the other factors. The first type provides a reliable assay, but, unfortunately, few laboratories have access to plasma from a patient congenitally deficient in factor V. The second type, in which the artificial factor V deficient plasmas are used, has given less reliable results. Preparation of the reagents is time consuming and the reactivity may often be unpredictable and variable. Thus, the need for a good artificial substrate remains.

The present assay method is based on the observation by Hjort[1] that Russell's viper venom even in the absence of Ca^{++} rapidly converts proaccelerin to accelerin, with subsequent inactivation of the accelerin formed. The Russell viper venom will be shown to specifically remove factor V from the substrate plasma by this mechanism. It also supplies (with phospholipid) the thromboplastic activity for the assay system.

MATERIALS

Normal Plasma.—Any normal, citrated (1 part 3.13 per cent sodium citrate dihydrate to 9 parts blood), platelet-free plasma (centrifuged for 30 minutes at 3,000 r.p.m., or 1,800 r.p.m. may be used.

Russell's Viper Venom in Cephalin Suspension.—Cephalin prepared according to the Seaport and co-workers'[2] modification of Milstone's method is diluted to optimal concen-

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tration for the particular preparation in Veronal buffer, pH 7.3.[3] Stypven* is dissolved in the cephalin suspension to a concentration of 1:100,000 (10 mcg. per milliliter).

$CaCl_2$.—For this system 0.035M $CaCl_2$ was optimal.

I. *Preparation of the Proaccelerin-Deficient Plasma Substrate.*—Equal parts of the Russell viper venom–cephalin suspension and normal platelet-free plasma are mixed and the pH of the mixture is adjusted to 8.5 ± 0.1 by addition of 1N NaOH. Since the ratio of glass surface to plasma volume affects the rate of accelerin destruction, the mixture is distributed in 10 ml. quantities in scrupulously clean 15 ml. test tubes. These are stoppered and placed in a water bath at 37° C. until the "blank time" reaches 60 to 70 seconds. The storage time is approximately 4 hours. The samples are then pooled and the pH is readjusted to 7.3 with 1N HCl. At this pH the "blank time" usually is a few seconds shorter. The reagent is stored in small aliquots at –20° C.

TABLE I. INFLUENCE OF PLATELETS, CEPHALIN, AND CHYLOMICRA ON INACTIVATION OF FACTOR V IN PLASMA INCUBATED WITH RUSSELL'S VIPER VENOM AT pH 8.5

INCUBATED TOGETHER WITH RUSSELL'S VIPER VENOM	"BLANK TIME" FOR SUBSTRATE AFTER 4 HOURS' INCUBATION (pH RETURNED TO 7.3; SECONDS)
Fasting, platelet-poor plasma	43.7
Fasting, platelet-poor plasma + cephalin	70.6
Fasting, platelet-rich plasma	19.2
Fasting, platelet-rich plasma + cephalin	35.4
Fatty meal, platelet-poor plasma	41.0
Fatty meal, platelet-poor plasma + cephalin	68.0
Fatty meal, platelet-rich plasma	18.2
Fatty meal, platelet-rich plasma + cephalin	31.1

Comments.—

a. *Plasma:* It is essential that the plasma used is free of platelets or platelet fragments because such material prevents the complete inactivation of the accelerin activity as shown in Table I. At present we cannot explain this effect. It does not seem to be a simple consequence of the proaccelerin which is adsorbed to platelets.[4] Chylomicra exert little, if any, influence on the rate of factor V destruction (Table I). There is consequently no need to specify the use of fasting plasma.

TABLE II. INFLUENCE OF RUSSELL'S VIPER VENOM CONCENTRATION ON RATE OF ACCELERIN DESTRUCTION IN PLASMA INCUBATED AT pH 8.5

RUSSELL'S VIPER VENOM CONCENTRATION	ORIGINAL "BLANK TIME" (pH 7.3; SECONDS)	"BLANK TIME" AFTER 4½ HOURS' INCUBATION (pH RETURNED TO 7.3; SECONDS)
1:40,000	8.4	121
1:100,000	10.7	93
1:500,000	18.5	70
1:1,000,000	21.5	62

b. *Russell's viper venom:* The specified Russell's viper venom concentration was selected for several reasons. (1) A certain minimum is required to assure complete conversion of proaccelerin to accelerin. A pilot study, however, showed that concentrations as low as 1:1,000,000 were able to do this within 2 minutes. (2) Although the natural plasma anti-accelerin plays a major role in the inactivation of accelerin,[1] the data of Table II show that Russell's viper venom contributes to this process in proportion to its concentration. The Russell viper venom concentration, therefore, must be high enough to secure rapid an-

*Burroughs Wellcome & Co., Tuckahoe, N. Y.

complete inactivation of accelerin. (3) A potent and stable thromboplastic activity for the assay is a further requirement of the Russell viper venom concentration selected. However, the concentration should not be so high that relevant clotting factors other than proaccelerin are significantly affected. Consideration of the data in Table II led to the choice of the 1:100,000 concentration as the most suitable, although 1:40,000 would probably also be satisfactory.

c. *Cephalin:* The thromboplastic activity of Russell's viper venom is incomplete without a phospholipid additive.[2] The data in Table I show that cephalin is also required for the complete destruction of accelerin when plasma is incubated with Russell's viper venom. An attempt was made to substitute an Asolectin* suspension for the cephalin, since many laboratories now use this commercially available material in order to avoid the preparation of the cephalin. Surprisingly, however, the incubation of a Russell's viper venom–Asolectin-plasma mixture for 4 to 5 hours resulted in very significant losses of the phospholipid activity. Even when 500 mg. per cent Asolectin was used (instead of the standard 100 mg. per cent concentration) the final phospholipid activity of the incubated mixture was considerably less than that of the cephalin preparation. The long clotting time could be corrected by addition either of fresh Asolectin or of cephalin (Table III). Since it would be difficult to secure optimal phospholipid concentration in the final product under such conditions, cephalin should be used for this assay.

TABLE III. DEFICIENCY OF PHOSPHOLIPID ACTIVITY IN SUBSTRATE PREPARED BY INCUBATION WITH ASOLECTIN; CORRECTION BY ADDITION OF FRESH ASOLECTIN OR CEPHALIN

FINAL DILUTION OF TEST PLASMA IN:	CLOTTING TIME FOR ASSAY OF 2 % NORMAL PLASMA (SECONDS)	
	ASOLECTIN—PLASMA–RUSSELL'S VIPER VENOM SUBSTRATE	CEPHALIN—PLASMA–RUSSELL'S VIPER VENOM SUBSTRATE
Buffer	33.1	22.0
Buffer + Asolectin	18.4	21.1
Buffer + cephalin	18.0	19.8

d. *pH:* The selection of pH 8.5 for incubation of the substrate mixture resulted from Stormorken's[5] finding that the rate of destruction of factor V in plasma increased with increasing alkalinity. Table IV shows the increasing "blank time" obtained with plasma–Russell's viper venom–cephalin mixtures incubated at different pH's. At pH 9.0, or above, fibrinogen is destroyed, as has also previously been reported.[5]

TABLE IV. INFLUENCE OF pH ON RATE OF INCREASE OF SUBSTRATE "BLANK TIME"

pH DURING INCUBATION	"BLANK TIME" OF SUBSTRATE AFTER 4 HOURS' INCUBATION AND RETURN OF pH TO 7.3 (SECONDS)
7.3	28.1
8.0	51.7
8.5	63.3

e. *Incubation time:* Incubation was continued until the "blank time" was 60 to 70 seconds. This was achieved in 3½ to 4½ hours.

II. *Performance of the Test.*—A 10 by 70 mm. test tube containing 0.4 ml. of the reagent is placed in the water bath for 3 to 10 minutes. Two-tenths milliliter of the diluted test plasma is added; *exactly* 2 minutes' preincubation is timed with a stop watch. The mixture is then recalcified with 0.2 ml. of the $CaCl_2$ solution and the clotting time is determined. The clotting time is converted to per cent factor V activity by means of a correlation graph prepared by serial dilutions of normal plasma (Fig. 1). Constant ionic strength and citrate

*Associated Concentrates, Inc., Woodside, L. I., N. Y.

concentration are maintained in all dilutions by the technique described by Hjort and associates.[3] For the "blank time," 0.2 ml. of dilution fluid II (200 ml. of Veronal buffer, 200 ml. of 25.66 mM. sodium citrate, and 600 ml. of 0.9 per cent NaCl solution) is used in place of diluted test plasma. The reason for the choice of a 2 minute preincubation time can be

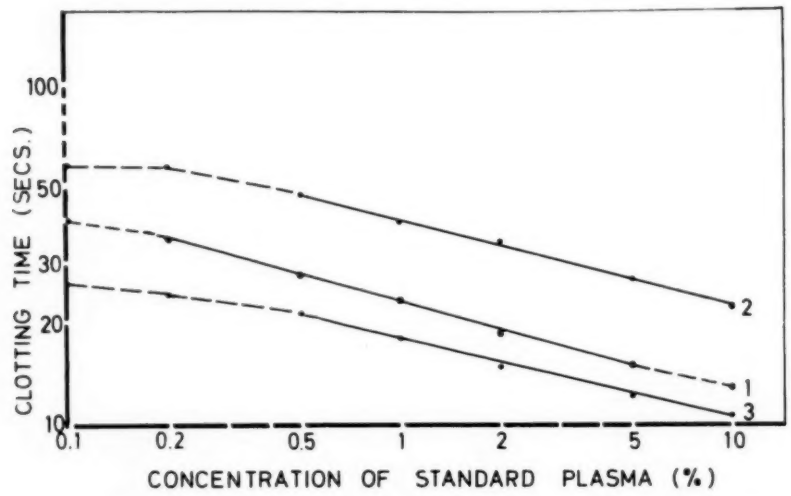

Fig. 1.—Standard dilution curves for 3 assay systems. *1*, Normal plasma–Russell's viper venom–cephalin substrate of this report; *2*, congenitally deficient plasma–brain thromboplastin substrate; *3*, congenitally deficient plasma–Russell's viper venom–cephalin substrate.

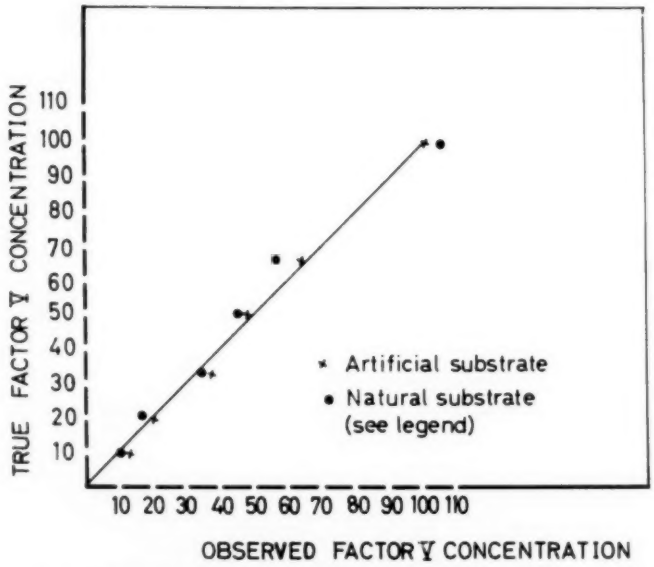

Fig. 2.—Dilutions of normal plasma assayed with 2 different substrates: normal plasma–Russell's viper venom–cephalin (*x*) and congenitally deficient plasma–brain thromboplastin (●).

understood by referring to Fig. 3. The clotting times of the assay system are shown when normal plasma, proaccelerin-deficient plasma, or a suspension of normal platelets was used as the source of factor V. Increasing incubation intervals were permitted between addition

of the test plasma to the substrate and addition of Ca^{++} to the system. The complete conversion of proaccelerin to accelerin takes place in less than 2 minutes in these, as well as in all other plasmas tested, as indicated by the shortening of the clotting time. Destruction of the accelerin prevails, as indicated by the lengthening of the clotting time. From these curves it is apparent that exactly 2 minutes' preincubation must be used, particularly if small quantities of factor V are being measured.

III. *Standard Curve.*—Normal standard plasma was serially diluted using the technique of Hjort and associates.[3] The correlation curve is shown in Fig. 1. It is a straight line on double logarithmic paper between the concentrations of 5 per cent and 0.2 per cent. For comparison, two other curves are shown in the same figure, both prepared using substrate plasma from Owren's[6] original patient who was congenitally deficient in factor V. One was made with human brain and the other with Russell's viper venom plus cephalin as the source of thromboplastic activity. The curve obtained with the artificial substrate is slightly steeper, and remains straight at lower concentrations of normal plasma than the other two. These observations are compatible with the evidence, discussed under sensitivity, that the artificial substrate contains less factor V activity than does the plasma of the patient congenitally deficient in facor V.

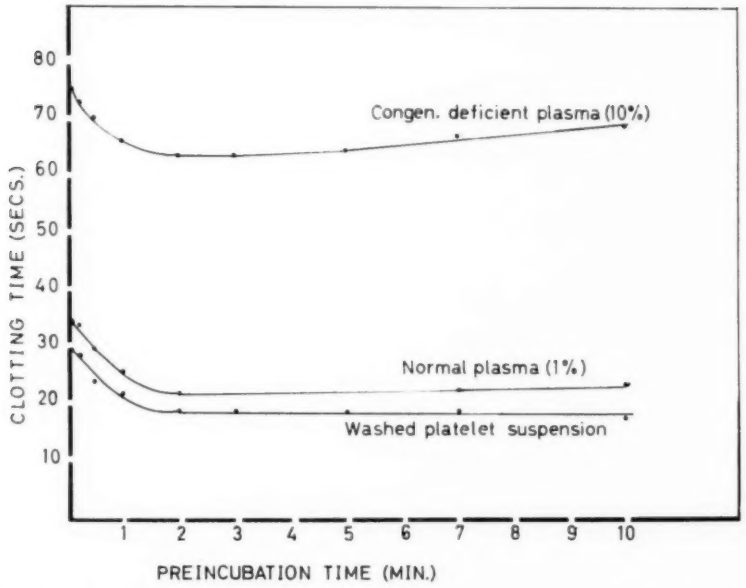

Fig. 3.—Change in factor V activity during preincubation with Russell's viper venom.

IV. *Specificity.*—Several tests were performed to assure that the long "blank time" was caused only by the deficiency of factor V and not by the destruction of any other essential factor. Thus, the addition of purified fibrinogen* to the system did not shorten the clotting time, which indicates that a significant loss or change in reactivity of fibrinogen had not taken place. The remaining factors of concern are only prothrombin and Stuart-Prower factor because Russell's viper venom and cephalin together provide the equivalent of tissue thromboplastin plus proconvertin.[2] The "blank time" could be completely corrected by adsorbed plasma or by plasma from patients on anticoagulant therapy; therefore, the substrate was not deficient in either prothrombin or Stuart-Prower factor. Several further observations confirmed this conclusion: a highly purified proaccelerin

*Kabi, Stockholm, Sweden.

preparation,[1] made from twice adsorbed plasma, was completely effective, and a concentrate of the adsorbable serum factors (prothrombin 55 per cent, proconvertin 500 to 600 per cent) was completely ineffective in correcting the "blank time." Furthermore, the substrate and its parent plasma were both tested in the Russell viper venom prothrombin assay[3] (Stuart-Prower factor–sensitive) and had exactly the same value. Finally, dilutions of normal plasma were assayed both in the new assay system and in the one based on congenitally deficient plasma (Owren's patient[6]) and brain thromboplastin (Fig. 2). The coincidence of the paired points confirms that both systems measure the same variable. It can be concluded that of the known clotting factors necessary in this system, the substrate is deficient only in factor V.

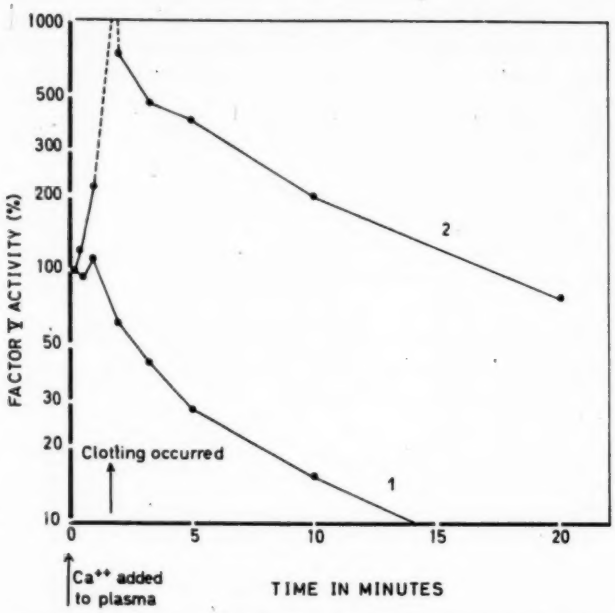

Fig. 4.—Change in factor V activity in recalcified plasma as measured with 2 different subtrates: *1*, incubated normal plasma–Russell's viper venom–cephalin, and *2*, congenitally deficient plasma-brain thromboplastin.

V. *Sensitivity.*—The sensitivity of the assay system will depend on the completeness of the factor V deficiency of the reagent. A test of the artificial substrate in an assay system which consisted of Russell's viper venom, cephalin, and plasma from the congenitally deficient patient showed that the "blank time" of the latter was not shortened. On the other hand, addition of the patient's plasma to the artificial substrate did shorten the clotting time. These findings show that there is less factor V in the artificial substrate than in the plasma of the patient congenitally deficient in factor V. Measurement of the patient's factor V level in this assay system gave a value of approximately 0.5 per cent. This is in agreement with the findings of Hjort and co-workers.[4] A second factor V–deficient patient had slightly less, 0.35 per cent.

That the plasmas of these patients actually have small quantities of proaccelerin may be confirmed by incubation of either plasma with Russell's viper venom for varying intervals before addition of Ca^{++}. The clotting time decreases during the first 2 minutes of incubation because of the conversion of the proaccelerin to accelerin, and then steadily lengthens, because of destruction of the accelerin so formed. This experiment appears in Fig. 3.

VI. *Stability.*—When stored in the liquid frozen state the reagent showed constant reactivity for at least 3 months, as checked by testing several dilutions of fresh plasma

from the same donor. There was, however, some increase in the "blank time" during storage, presumably because of further deterioration of the small residuum of factor V. Since the level at the beginning of storage was vanishingly low when compared with that of the test plasma, the further decline did not affect the standard curve.

VII. *Comparison Between the New Method and the Method Based on Congenitally Deficient Plasma and Thromboplastin.*—Factor V activity increases greatly during coagulation.[7] This increase is easily demonstrated when normal plasma is recalcified and its factor V activity is assayed, at intervals, in the system based on the use of brain thromboplastin and congenitally deficient plasma. The new method, however, measures all the potential factor V activity in the test sample, and the increased activity therefore does not show up. (Fig. 4.)

Plasmas from 46 normal subjects were tested in both systems. It will be seen from Fig. 5, that there is an almost even distribution around 100 per cent in both systems, but that the range is narrower in the new assay.

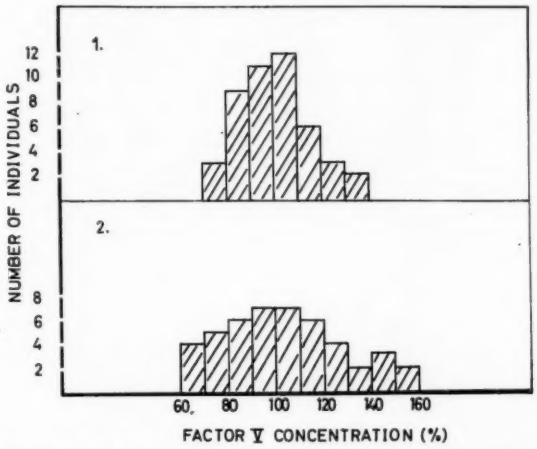

Fig. 5.—Distribution of plasma factor V levels for 46 normal individuals using 2 different substrates: *1*, incubated normal plasma–Russell's viper venom–cephalin, and *2*, congenitally deficient plasma–brain thromboplastin.

DISCUSSION

The assay described in this report differs from the assay based on congenitally deficient plasma in 2 respects. First, the latter employs plasma from congenitally deficient subjects as substrate; this one employs a plasma artificially depleted of factor V. Although the naturally deficient plasma contains more factor V than the artificially depleted one, its sensitivity is quite satisfactory. The problem is, therefore, not the sensitivity, but the scarcity, of congenitally deficient plasma. Second, two fundamentally different types of thromboplastin are used. It seems clear from Fig. 3 that a Russell's viper venom assay converts all of the proaccelerin in a test plasma to accelerin before the latter is measured, but this is not the case with a brain thromboplastin assay. It is probable that with brain thromboplastin, variable fractions of the original proaccelerin are converted before clotting. This difference follows from the fact that Russell's viper venom can effect the conversion in the absence of Ca^{++}, whereas brain thromboplastin can effect it only after recalcification of the plasma.

Thus, in the latter case, conversion of prothrombin to thrombin, of fibrinogen to fibrin, and of proaccelerin to accelerin all occur simultaneously, and a fibrin clot may well appear before the last-mentioned conversion has been completed. The clotting time would then not reflect all of the potential accelerin in the test sample. As a result, one would theoretically expect a Russell's viper venom assay to provide better accuracy and reproducibility than a brain thromboplastin assay. Fig. 5 shows that when this question was investigated by making factor V estimations in 46 normal individuals with both the brain thromboplastin–congenitally deficient plasma method and the Russell's viper venom–cephalin-normal plasma method, the range was narrower with the Russell viper venom system, which suggests that the hypothesis may be true.

SUMMARY AND CONCLUSIONS

An artificial substrate for factor V assay is described. It is prepared by incubation of a mixture of Russell's viper venom, cephalin, and normal plasma, as a result of which the proaccelerin of the plasma is specifically destroyed.

The system has 3 principal advantages: it does not require congenitally deficient plasma; it provides a substrate with predictable and reproducible characteristics after only 4 hours' incubation; and, because of its Russell's viper venom–cephalin thromboplastin it permits complete conversion of proaccelerin to accelerin, and, therefore, complete measurement of all the factor V activity in any test sample.

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STUDIES ON PASTEURELLA TULARENSIS

I. Effect of Duolite Treatment on Growth Promoting Properties of Blood and Plasma

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HUDDLESON[1] reported that treatment with cationic exchange resins, particularly Duolite C-3^{H+}, markedly inhanced the ability of certain blood specimens to support the growth of Brucella organisms. Huddleson concluded that "The growth of brucella cells in a mixture of culture medium (peptone type) and blood is not due to the growth promoting constituents in the medium but to the presence of an agent or agents that inactivate the bacterial growth inhibiting factor usually present in normal blood. The blood then becomes an excellent culture medium." He demonstrated that when blood of cow, horse, or man was treated with sufficient cationic exchange resin in the hydrogen form to lower the pH to the range of 6.0 to 6.2, it became a good culture medium, promoting rapid growth of small numbers of organisms of test strains of all species of Brucella. Further, if the procedures described were carried out in a closed container (i.e., vaccine bottle with rubber diaphragm stopper), the addition of carbon dioxide (CO_2) for the growth of CO_2-dependent strains of *Brucella abortus* was unnecessary.

In view of the facts that *Pasteurella tularensis* in certain respects resembles organisms of the Brucella group and is difficult to isolate consistently from the blood stream of animals and men known to be infected, particularly in the early stages of disease, it occurred to us that similar methods might be employed for studies with this organism. It was hoped that investigations into the basic mechanisms involved in the enhancement of bacterial growth from small inocula might give useful information needed for the development of selective media for the isolation of *Past. tularensis* from clinical specimens (other than blood) which normally contain flora which rapidly overgrow the plates when present methods are used.

MATERIALS AND METHODS

Preparation of Blood and Plasma.—The resin Duolite C-3^{H+*} is a phenolic matrix with methylene sulfonic acid as the functional group; it was used in all experiments included in this report. Preliminary work with the sodium form of Duolite C-3 and also with certain other resins has been started and will be discussed in a later publication.

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The resin was washed and titrated with each batch of blood or plasma as prescribed by Huddleson.[1]

After titration, the determined amounts of Duolite were weighed out and placed in 60 ml. vaccine bottles to which were added 2.0 ml. of a 5 per cent sodium citrate solution (referred to hereafter as citrate). The bottles were plugged with cotton and autoclaved for 20 minutes at 121° C. and allowed to cool to room temperature. The cotton plugs were then replaced with sterile rubber diaphragm vaccine stoppers.

With the use of a syringe and needle, 10 ml. aliquots of test blood were introduced into the bottles, and the contents were mixed thoroughly by rotation.

Blood samples were pooled before titration and used within 72 hours after collection. Plasma, when used, was obtained by centrifugation of defibrinated whole blood. Procedures for preparation and testing were the same in both cases.

Initially, 2 types of control bottles were used: one with 10 ml. blood plus 2 ml. citrate, without Duolite, and the other with 12 ml. defibrinated blood, without either citrate or Duolite. It was found that the growth of *Past. tularensis* was inhibited by citrate alone, so this control was eliminated.

Bacterial Suspension for Inoculum.—The organism used in all experiments was the streptomycin-sensitive SCHU S4 strain of *Past. tularensis.* Plates of glucose cystine blood agar (GCBA; prepared with Bacto-cystine* heart agar and 5 per cent defibrinated sheep blood) were streaked heavily with the test organism and incubated for 24 hours at 37° C.

A concentrated bacterial suspension was made by emulsifying the growth from one or more of these plates in 2 to 5 ml. amounts of tryptose-saline (0.1 per cent Bacto-tryptose* in 0.5 per cent saline, adjusted to pH 7.2 to 7.4). This suspension was adjusted to approximate a predetermined density as measured by the Bausch and Lomb Spectrotronic 20 colorimeter. Light transmittance was determined at 4 wave lengths for the initial calibration and for subsequent preparations. The adjusted suspension was diluted serially to that concentration which would give the desired number of organisms in 0.2 ml., the volume used as inoculum for all tests. All density adjustments and dilutions were made with the tryptose saline solution. A total inoculum per bottle of 50 to 100 cells was employed in all experiments, with the exception of 2 tests with 5 to 10 cells.

Experimental Procedures.—Test and control bottles were inoculated with 0.2 ml. aliquots of the bacterial suspension. Similar aliquots were plated on 4 or more GCBA plates to determine the actual number of cells used for inoculum. The initial dosage was calculated as the number of organisms per milliliter of culture medium, i.e., 6 organisms in 12 ml. of fluid was expressed as 0.5 organism per milliliter.

The bottles were incubated at 37° C. in an Aminco-Dubnoff shaker incubator equipped with a water bath. During the incubation period the shaker was operated at 90 cycles per minute. All plates and those duplicate bottles in experiments designed to compare shake with static cultures were incubated in a water-jacketed incubator at 37° C. Sufficient duplications of test and control bottles were prepared to allow for removal of samples for examination at 12, 24, 48, and 72 hours.† The method of examination was as follows: four 0.2 ml. aliquots were removed from each bottle with a sterile syringe and needle and inoculated onto GCBA plates. A glass spreading rod was used to distribute the inoculum over the agar surface. If confluent growth was expected, serial dilutions of the material were made in tryptose-saline and 0.2 ml. aliquots of the dilutions were similarly plated. These plates were incubated for 72 hours before final counts were made.

Most of the work was performed in the preceding manner, although several variations were added as time permitted: (1) comparison of static with shaker incubation, (2) comparison of plasma with whole blood, with and without Duolite treatment, and (3) comparison

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†In 1 experiment, in which repeated samples for plating were taken from a single bottle at the various time intervals, counts essentially comparable to those of other tests were obtained at 72 hours; however, the 24 and 48 hour counts were consistently lower than those obtained by the method described.

of the effect on growth obtained when Duolite was removed after initial treatment of the specimen, but prior to inoculation, with that obtained in cultures in contact with the resin throughout the incubation period.

RESULTS

A summary of results of the effect of Duolite treatment on the growth of *Past. tularensis* in rabbit, sheep, and human blood is given in Table I. The recoveries given in the table are the arithmetic means of average plate counts obtained in 3 trials with sheep blood, 5 trials with rabbit blood, and a single trial with human blood. Both rabbit and human blood are superior to that of sheep for growth of *Past. tularensis.* Duolite treatment markedly enhances the growth of the organism in blood of all 3 species, but does not obliterate the species difference.

TABLE I. COMPARISON OF MEAN RECOVERIES* OF PAST. TULARENSIS IN BLOODS FROM DIFFERENT SPECIES AT VARIOUS INCUBATION TIMES
(Mean inoculum: 5.7 organisms per milliliter of culture medium)

TEST BLOOD	DUOLITE	TIME OF INCUBATION (HOURS) 12	24	48	72
		Mean recovery organisms/ml. of culture fluid*			
Sheep	None	1	38	8	510
Rabbit	None	43	530	1,600	100,000
Human	None	—	60	1,600	12,000
Sheep	Present	5	25	500	50,000
Rabbit	Present	87	80,000	4.0×10^9	5.7×10^9
Human	Present	—	11,000	2.5×10^6	1.0×10^8

*Recoveries in number of organisms per milliliter of medium expressed as the arithmetic mean of available mean counts.

Of special interest, are the data in Table II which show the results of 2 experiments with a smaller inoculum. There was evidence of continued multiplication of the organisms in untreated rabbit bloods inoculated with approximately 70 organisms. However, when the initial concentration was reduced to 6 cells in 12 ml. blood, there appeared to be some multiplication within the first 12 hours but none thereafter. In Duolite treated rabbit bloods examined in parallel, approximately the same counts were obtained after the first 12 hours, regardless of size of inoculum.

With sheep and rabbit bloods, where the results of 3 and 5 replicate experiments were available, remarkably little variation from one experiment to another was observed, despite the use of different batches of blood and the experimental error inherent in procedures involving sampling and counting. For these

TABLE II. EFFECT OF DUOLITE TREATMENT AND SIZE OF INOCULUM ON RECOVERY OF PAST. TULARENSIS FROM RABBIT BLOOD

DUOLITE	INOCULUM	TIME OF INCUBATION (HOURS) 12	24	48	72
		Mean recovery: organisms/ml. of medium			
None	5.7/ml.	43	530	1,600	100,000
None	0.5/ml.	20	3	6	7
Present	5.7/ml.	87	80,000	4×10^9	5.7×10^9
Present	0.5/ml.	1	13,000	2.1×10^9	5.3×10^9

experiments, it was not feasible to plate at identical time intervals; consequently, results at 24, 48, and 72 hours varied as much as 2 to 4 hours either way from experiment to experiment. While the generation time of *Past. tularensis* in Duolite treated blood cannot be calculated accurately from available data, it appears to be between 60 and 90 minutes in the period between 24 and 48 hours; counts made then could be expected to vary as much as tenfold or more in any 2 to 4 hour interval. Counts made at 72 hours in replicate experiments never varied as much as tenfold, and those at 24 and 48 hours always fell within the limits of variation which could be accounted for by experimental error.

TABLE III. COMPARISON OF STATIC VS. SHAKER INCUBATION USING DUOLITE TREATED RABBIT BLOOD
(Inoculum: 0.5 organism per milliliter)

INCUBATION CONDITION	RECOVERY ORGANISMS/ML. OF CULTURE FLUID AT INDICATED TIME INTERVALS		
	24 HOURS	48 HOURS	72 HOURS
Static (water jacketed incubation 37° C.)	$>1.5 \times 10^4$	2.5×10^8	2.5×10^8
Shaker (Amineo-Dubnoff 37° C. water bath, 90 strokes/min.)	$>1.5 \times 10^4$	4.2×10^9	5.7×10^9

Tables III and IV present the results of variations from the standard procedure. It appears that counts obtained in shake culture of Duolite treated rabbit blood are about 10 times higher than those obtained from comparable preparations incubated under static conditions.

TABLE IV. COMPARISON OF RABBIT BLOOD AND RABBIT PLASMA WITH DUOLITE PRESENT AND DUOLITE REMOVED AFTER TREATMENT
(Inoculum: 0.5 organism per milliliter. Shaker incubated.)

MEDIUM	RECOVERY ORGANISMS/ML. OF CULTURE FLUID AT INDICATED TIME INTERVALS		
	24 HOURS	48 HOURS	72 HOURS
Blood + Duolite	$>1.5 \times 10^4$	4.2×10^9	5.7×10^9
Blood + Duolite; Duolite removed	$>1.5 \times 10^4$	$>5.0 \times 10^7$	5.8×10^9
Plasma + Duolite	5	>5	>5
Plasma + Duolite; Duolite removed	1.7×10^3	1.5×10^7	1.9×10^9

The data given in Table IV on the removal of Duolite before incubation are of theoretic, as well as practical, interest. There is apparently no significant difference in counts obtained from whole blood under the 2 experimental conditions, Duolite present and removed; however, it would appear that the resin must be removed from plasma prior to inoculation of the organism and incubation of the culture if one is to achieve enhancement of the growth of *Past. tularensis* in this medium. In this connection it is of interest to note that the amount of Duolite required to bring the pH of 10 ml. of rabbit plasma to the

desired range of 6.0 to 6.2 is almost exactly ½ that amount necessary for the same adjustment of whole blood. No satisfactory explanation for this observation can be given at the present time. The possibility that wider variations of pH might occur during the incubation of plasma medium in the presence of a cationic exchange resin than after its removal, has been ruled out. After the initial observation, a second experiment was conducted in which the hydrogen ion concentration was determined at each plating time. The pH values in blood and plasma bottles, regardless of the presence or absence of Duolite, were identical, as were the results of the 2 trials. Further investigations on this point are under way.

DISCUSSION

The work reported here clearly demonstrates that blood of sheep, rabbit, and man, and rabbit plasma treated with Duolite C-3^{H+} are excellent culture media for small inocula of *Past. tularensis*. It also confirms the original observations of Huddleson who was working with Brucella.

The possible applications of this method to clinical bacteriologic problems are obvious. We have not had the opportunity to evaluate the method described as a blood culture technique for diagnosis of tularemia in man. However, this technique (adapted to use with 2 ml. of blood) has been successfully applied to the detection of bacteremia in monkeys challenged with *Past. tularensis*.[2] Positive blood cultures were obtained in 24 of 36 animals examined; 10 of the 12 failures were in animals which had been vaccinated with a living, attenuated strain of *Past. tularensis* prior to challenge and which were put on drug therapy 8 to 16 hours after the appearance of the first rise in temperature. The detection of bacteremia in 5 animals 8 to 16 hours prior to the first recorded fever and in 8 others simultaneously with the first temperature rise was of special interest. Although it was not possible to examine aliquots from the same animals using the conventional methods for blood culture, attempts to isolate tularemia organisms from blood under comparable circumstances in previous work have rarely proved successful. It is hoped that the indicated controlled studies can be done in the near future.

Results of the comparison of the growth promoting properties of blood from the 3 species studied parallel exactly, and in the expected reverse order, the findings of Stanziale,[3] who studied the in vitro bactericidal activity of blood for *Past. tularensis*. He found that sheep blood had considerably more bactericidal activity than blood of either humans or rabbits and that human specimens were slightly more active than were rabbit specimens. Duolite treatment, although it markedly enhanced growth in all cases, did not obliterate this species difference.

The mechanism involved in the observed enhancement of growth of *Past. tularensis* in Duolite treated blood remains to be elucidated. Investigations on this point are under way.

Attempts to identify by various methods an inhibitory factor actually removed from blood or plasma by the resin have been unsuccessful. However, the findings appear to be in agreement with preliminary work reported by

Huddleson, with one major exception: we have found the sodium form of Duolite C-3 (prepared by treating the commercially available product with sodium chloride) to be equally, or more, effective than the hydrogen form, provided that citrate is omitted in the preparation procedure. In these experiments defibrinated blood was used and the resin was moistened with a small amount of physiologic saline prior to autoclaving.

Other studies in which oxygen consumption determinations are made with both the Warburg respirometer and the plate count technique strongly suggest that factors which could theoretically affect the oxidation reduction (O-R) potential of the media and perhaps the metabolism of sulfydryl compounds may be involved. An apparatus, called an Eh-Stat, with which O-R potential can be measured, and stabilized, at a specific level has been constructed and is now in use to investigate this point. A description of the Eh-Stat will be published later.

The repeated observation that the enhancing effect of Duolite treatment of plasma can be obtained only if the resin is removed from the specimen prior to inoculation with the test organism (Table IV) is of considerable theoretic interest, especially since the final hydrogen ion concentration of the blood and plasma is identical. This observation clearly demonstrates that the resin effects some change in the medium which is not reflected in measurement of hydrogen ion concentration alone, and suggests that the presence of erythrocytes serves to stabilize this change, whatever it might be.

SUMMARY

Studies with the streptomycin-sensitive SCHU S4 strain of *Pasteurella tularensis* have demonstrated that treatment with Duolite C-3^{H+} markedly enhances the growth promoting properties of blood and plasma. These results are similar to those obtained by Huddleson who used Brucella species as test organisms.

The enhancing effect of resin treatment has been demonstrated in blood specimens taken from rabbit, sheep, and man.

Untreated rabbit and human bloods are superior to sheep blood as media for growth of *Past. tularensis.* Growth in rabbit blood is somewhat better than in human blood. These observations are in agreement with work done by others on the bactericidal activity of bloods of these species for the organism under study.

Enhancement of growth by Duolite treatment of plasma could be obtained only if the resin was removed before inoculation and incubation.

The mechanism involved in the observed enhancement of growth of *Past. tularensis* in blood and plasma specimens treated with Duolite remains to be elucidated. Certain factors which might be involved are discussed.

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A pH STAT SUITABLE FOR CONTROL OF pH IN BACTERIAL CULTURES

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SEVERAL devices have been constructed for the purpose of maintaining constant pH values in active biologic systems.[1] None of these has been suitable for bacteriologic work in which avoidance of contamination as well as control of pH is required. Described in this article is an economical and satisfactory system for the maintenance of the pH of bacterial cultures at constant and controlled values. It has been constructed so that many separate cultures may be monitored by a single unit.

The unit utilizes as its basis an automatic titrator.* This titrator is an electronic pH meter with an output which operates a solenoid valve controlling a buret. The valve remains open as long as there is an error signal produced by the difference in the pH of the solution and the end point set on the instrument. This apparatus in its commercially obtainable form is suitable for the monitoring and control of a single study unit. It is not suitable for the regulation of multiple units.

Although multiple titrators may be used to regulate multiple units and will provide continuous monitoring, such monitoring of each unit is not necessary when the changes in pH are small or occur slowly. It should thus be feasible to transfer the monitoring system to various study units in such a way that automatic surveillance of each unit is possible.

The apparatus to be described uses an inexpensive electric clock plus easily obtainable relays, pilot lights, jacks, and plugs. With this system, the automatic titrator monitors each culture in succession every 5 minutes or at any interval that is desired. Manifestly, the frequency with which each culture is monitored as well as the number of cultures that may be monitored may be changed by an alteration of the timing clock.

The apparatus consists of 3 parts: the automatic switch, the paired connecting relays, and the monitoring unit (Fig. 1). The automatic switch is a 110 V A.C. 60 cycle electric clock, the hands of which are removed. In place of the minute hand a lucite arm with 2 connected copper fingers is substituted.

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*Radiometer TTT 1A.

One finger rides on a buss wire ring set in lucite. The ring in turn is connected to one side of a 110 V line. The other finger rides on an interrupted ring each segment of which corresponds to 4 minutes' motion of the minute hand, with a 1 minute space between segments. Various segments of the circle may be wired together, if it is desirable to change the sequence or total time of monitoring of individual units. When contact between the 2 rings is made, 110 V A.C. is fed through the energizing coil of the pair of connecting relays wired to that segment. This actuation is shown by a pilot light across the coil. The relays are of 2 types: one is a DPDT relay* which, when actuated,

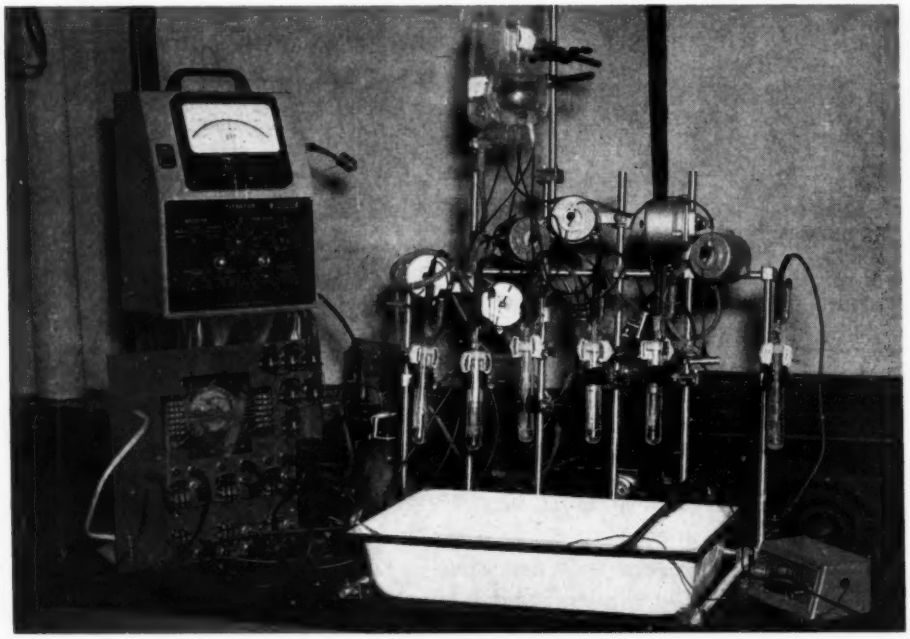

Fig. 1.—Complete apparatus. The titrator is above the switch and the relay circuit is on the left. The culture tubes and solenoids are on the right. The water bath, controlled by the adjacent thermostat, may be raised.

closes the circuit for the 2 portions of the measuring electrode system. In parallel with this is a SPDT relay† which connects the appropriate solenoid valve to the output of the titrator. It was found necessary to use 2 separate relays rather than one 3PDT relay; the proximity of a 120 V D.C. connection on the same relay alters the meter reading, controlled by the 100 mV D.C. connections.

The monitoring unit consists of the Radiometer-MV-1 magnetic valve, the GK-202 1A measuring electrode, the titrant feed and air mixing systems, and the buffer adjustment (Fig. 2). Connections from the monitoring unit to the

*Potter & Brumfield type KA14AY.
†Potter & Brumfield KA5AY.

connecting relays are made with standard telephone jacks and plugs, as are connections from relays to titrator.

Titrant is fed to all units from a common receptacle which is conveniently made from a 500 ml. round bottom flask into which 6 glass outlets were blown. Lines connecting the titrant pass through the solenoid valve and connect to hypodermic needles with plastic adaptors. These needles and similar ones connected to an air line are passed through polyethylene bottle caps in which are mounted the long-stemmed electrodes. The plastic tubing conducts air and titrant to the solution, as shown below. The lip of the bottle cap fits over the edge of the test tube which contains the study material. This overlap,

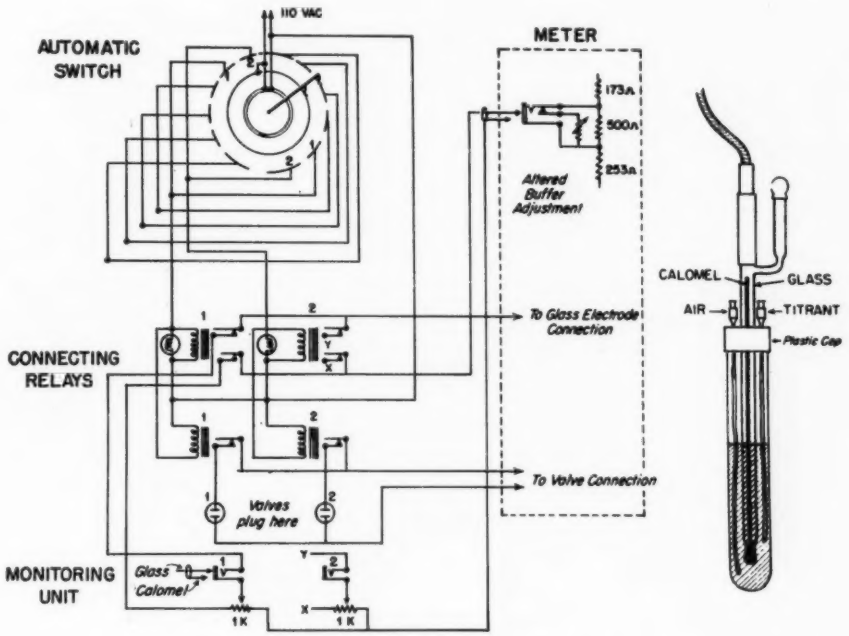

Fig. 2.—Line drawing of the switch, relay, and monitoring unit, and diagram (right) of the individual culture unit.

which resembles that in a petri dish, is successful in maintaining sterility of the system, when used in conjunction with the positive pressure air flow. Air is blown through a filter flask filled with absorbent cotton to free it of dust particles.

As in all electrical pH meters there is a buffer adjustment on this instrument to compensate for the variation of individual electrode pairs. Adjustment is made by altering the resistance between ground and the point in the circuit at which the measuring signal is applied. As shown in Fig. 2, this resistor is automatically disconnected when the switching circuit which is described is utilized, and the resistor attached to each monitoring unit is substituted in its place. This allows compensation to be set for each electrode pair and left unaltered during the later functioning of the machine.

With the system employed, only one final pH value may be set for all of the units controlled, and in all units this pH must be approached from the same direction, although from any initial level in that direction. It has been possible to maintain pH values with a variation of less than 0.1 unit during prolonged periods of multiplication of bacteria in culture media.

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LABORATORY AND CLINICAL EVIDENCE OF THE RELIABILITY OF THE ALKALINE-INCINERATOR METHOD OF SERUM PROTEIN-BOUND IODINE MEASUREMENT

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SANDELL and Koltoff,[1] in 1937, described a phenomenon which very greatly increased the sensitivity and accuracy of the measurement of iodine. In 1940, Chaney,[2] then Chief Chemist of the Los Angeles County Hospital, successfully applied this principle to small samples of serum. As a result, the clinical use of this test began in southern California.

Salter,[3] in 1944, after a great deal of work with older chemical techniques, reported the values found in man and small animals with this method and remarked, "indeed, it was suggested by Chaney (1940) that this reaction might be applied to biological materials." Taurog and Chaikoff,[4] in 1946, evaluated various methods and concluded that the method of Chaney was satisfactory and reliable. In 1948, Barker[5] described an improved digestion procedure. In 1950[6] we reviewed our experience with the blood protein-bound iodine (PBI) determination as a routine procedure. This article was based on clinical study in the Los Angeles County Hospital, in which Dr. Chaney had introduced his method, and referred to the findings in several hundred patients. In 1951, Barker[7] introduced the dry ash procedure, and stated that:

> "The value of plasma protein-bound iodine (PBI) measurement in the evaluation of thyroid disease has been well established, but it has not yet assumed its deserved place in the clinical armamentarium because of the technical difficulties involved in distillation procedures. Modification of the alkaline-incineration technique has resulted in the evolution of a method for PBI determinations which is *practicable for a clinical laboratory.*"

MATERIALS AND METHODS

Because of the clinical importance and reliability of this test, it is used extensively by physicians in southern California. The private clinical chemistry laboratories perform many thousands of such tests annually. In the Los Angeles County Hospital Laboratory as many as 6,000 tests may be done a year. In addition, the private laboratories receive specimens from all over the United States, and often from overseas. The validity of

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determinations on such specimens is made possible by the stability of the thyroxin even when the serum is not kept cold. As further evidence of the recognition of the reliability of the test, research projects in various parts of the United States, probably for economic reasons, send sera to Los Angeles for determination of the PBI.

TABLE I. GROUP I DUODECIMAL DETERMINATIONS OF EACH SERUM FROM 15 PATIENTS WHOSE ORIGINAL PBI VALUE RANGED FROM 0.6 TO 3.8 μG PER CENT

PATIENT	LABORATORY A		LABORATORY B		LABORATORY C		STANDARD DEVIATION
	RUN 1	RUN 2	RUN 1	RUN 2	RUN 1	RUN 2	
T. J.	1.1	1.1	1,3	1.6	1.1	0.9	0.33
	1.3	1.6	1.0	1.6	0.6	0.7	
N. S.	1.7	2.0	1.0	—	1.3	—	0.44
	1.9	1.8	1.1	—	0.9	—	
A. W.	2.1	2.2	1.5	1.4	1.4	1.9	0.37
	2.2	—	1.6	1.4	1.1	1.7	
S. L.	1.5	1.4	1.6	—	2.2	—	0.36
	1.5	1.4	1.5	—	2.3	—	
R. S.	1.6	1.8	1.5	1.5	1.6	1.4	0.49
	2.6	2.8	1.7	1.5	1.3	1.2	
J. S.	2.4	2.2	1.7	2.7	1.8	2.4	0.32
	2.4	2.3	2.1	2.4	2.2	2.8	
M. R.	3.3	3.7	3.6	3.6	3.4	3.1	0.21
	3.8	3.5	3.7	3.6	—	—	
S.	2.0	1.9	1.6	1.8	2.3	1.9	0.32
	1.9	1.7	1.7	2.0	1.6	1.0	
R. B.	2.4	2.1	2.3	2.5	2.4	2.5	0.24
	2.5	2.4	2.4	2.5	2.1	1.7	
S. M.	2.4	2.1	2.3	2.5	2.4	1.7	0.29
	2.5	2.4	2.4	2.5	2.1	1.7	
A. G.	2.4	2.4	1.7	2.0	1.5	2.1	0.56
	3.2	3.0	3.0	2.0	1.4	1.6	
H. N.	3.0	2.9	3.1	2.3	2.8	2.5	0.27
	3.0	3.0	3.3	2.7	2.7	2.9	
B. M.	2.5	2.0	2.2	1.6	3.3	2.9	0.45
	2.2	2.2	1.8	1.6	2.7	1.9	
R. M.	2.3	2.3	2.4	2.2	3.0	3.2	0.52
	2.3	2.3	2.4	2.2	3.0	3.3	
B. I.	2.4	2.9	2.3	1.7	2.1	3.4	0.42
	2.4	2.7	1.9	2.6	2.3	2.3	

Opinion that the test is unreliable, however, has been expressed by some of the most eminent authorities in thyroidology in England and the United States. Bauer[8] stated:

> "The levels of circulating protein-bound iodine (I^{127}) can be chemically determined, and represents a direct and reliable measure of thyroid hormone. *Unfortunately, the laboratory method is long, technically difficult, and subject to serious error through contamination by any source of iodine (air, glass, chemical).* Most clinics are not able to solve the problem of contamination and have abandoned this procedure as a routine test of thyroid function."

As eminent an authority as Beierwaltes[9] said, "*the serum PBI determination is so difficult to perform with consistent accuracy that its use is largely limited to University Laboratories.*"

The contradiction between the confidence in this test shown by thousands of physicians and scientists throughout the country and the distrust exhibited by many authorities has led us to test our own procedures by means of a cooperative blind study carried out by 3 experienced laboratories, called hereafter A, B, and C. In addition, since much of our confidence in the test results from years of experience in known individual cases, some of these data from the clinical research laboratory of one of us (P. S.) are presented. Thus we have 4 groups of technicians whose work is to be compared.

1. *Laboratory Studies of the Reliability of the PBI Test.*—

a. *Determinations of unknowns in duodecimate aliquots:* Blood was taken from 3 groups of patients known to be hypothyroid, euthyroid, and hyperthyroid, as follows: Group I, 15 patients whose original serum PBI ranged from 0.6 to 3.9 μg per cent; Group II, 9 patients whose original serum PBI ranged from 4.0 to 7.9 μg per cent; and Group III, 9 patients whose original serum PBI ranged from 8.0 to 21.6 μg per cent.

A large blood specimen from each of the 33 patients was divided into 6 aliquots (total 198). Each laboratory received 2 samples of the same patient's blood given different assumed names. Each of these was done in duplicate and both results were reported. Thus, 12 determinations were made on each serum. All determinations which were done are reported; a few analyses were lost because of breakage, etc. Each aliquot was coded with common names and mailed to the laboratories. To prevent the matching of aliquots by technicians, the second aliquot was sent after the first had been reported, similar initials on different samples were avoided, and similar PBI samples of different sera were sent at the same time.

Results of the intra- and interlaboratory crosschecks on blind samples are shown in Tables I and II. The last column gives the standard deviations calculated from all the determinations on each sample. The standard deviations so calculated thus include the variability occurring within runs, between days, and between laboratories. In actual practice these laboratories run unknown samples in duplicate and report the average. This would reduce the standard deviations to some degree. It is also apparent, as expected, that the variation within any one laboratory is less. Analysis of the data indicates that the results of Laboratory A ran slightly, but significantly, higher than the results of the other 2 laboratories, indicating either higher recovery of iodine or a somewhat different standardization of the test. Nevertheless, the agreement between laboratories is believed to be quite acceptable. The differences which did occur are well within the clinical order of magnitude involved and would not produce erroneous diagnosis. Perhaps fortuitously, there is not a single instance of intergroup overlapping.

b. *Determinations of known internal control sera:* One of the guides to reliability used by these laboratories is that a serum of established value was included in each run. These usually contained 25 or more specimens. The values found by using such a normal control* sample for 3 months are given in Table III. These are the actual values obtained by the technician† while testing successive batches of clinical samples to which this known control serum was added. The average of all runs was 5.4 μg per cent with a standard deviation of 0.33. A single value of 6.5 is included in the calculation, although in actual practice such a discrepancy would warrant repetition of the whole run. The 95 per cent confidence limits are approximately 0.5 μg per cent when the test is performed in duplicate. This represents the expected error obtained on normal specimens in a well-controlled laboratory.

2. *Clinical Experience Indicating the Reliability of the Serum PBI Test.*—

a. It is usual to make several repetitions of the test in patients with athyreotic myxedema. Such repeated values before treatment from 15 patients are shown in Table IV.

*Hyland Laboratories, Los Angeles, Calif.

†We are happy to take this opportunity to acknowledge the technical assistance of Maria Deering who has performed several thousand serum PBI determinations during the past 5 years.

b. One of the faculty members (Dr. George S.) took triiodothyronin in daily dosage adequate to suppress his own thyroid gland. This produced a *chemical* condition simulating the extremely low values found in myxedema. Seventeen determinations were made from July 13, 1953, to April 19, 1956. Eleven of these were made within 24 hours on April 14 after administration of a single dose in an experiment to observe any occurrence of accumulation of triiodothyronin in the blood.

TABLE II. GROUPS II AND III DUODECIMAL DETERMINATIONS IN 2 GROUPS OF 9 PATIENTS WHOSE PBI VALUES RANGED FROM 3.8 TO 7.0 AND FROM 12.0 TO 21.6 μG PER CENT, RESPECTIVELY

PATIENT	LABORATORY A		LABORATORY B		LABORATORY C		STANDARD DEVIATION
	RUN 1	RUN 2	RUN 1	RUN 2	RUN 1	RUN 2	
Group II.—							
L. G.	4.5	4.5	4.6	4.6	4.6	4.9	0.39
	4.6	4.9	—	—	3.8	3.8	
N. W.	4.7	4.9	5.0	4.6	4.8	—	0.19
	5.0	5.0	4.8	4.8	4.7	4.4	
S. W.	5.3	5.2	4.0	—	4.0	—	0.66
	5.0	5.3	3.8	—	4.1	—	
J. F.	4.7	4.6	4.8	5.4	4.7	4.7	0.39
	4.5	4.4	5.1	5.0	4.3	3.9	
M. V.	5.4	5.6	5.4	5.2	4.7	—	0.37
	5.2	5.4	5.0	4.9	4.7	4.4	
J. K.	5.7	5.7	4.8	4.7	4.8	—	0.40
	5.4	5.5	4.9	4.8	5.0	5.0	
F. G.	5.7	6.1	5.4	5.4	5.9	—	0.55
	5.9	6.1	4.9	—	4.5	—	
M. G.	5.9	5.3	4.5	—	5.2	—	0.59
	6.1	6.2	5.4	—	4.9	—	
M. T.	6.7	6.1	5.0	—	4.9	—	0.76
	7.0	6.4	5.9	—	5.6	—	
Group III.—							
S. A.	11.9	12.1	12.3	13.1	10.8	10.4	0.75
	12.2	12.6	12.3	12.2	11.8	11.4	
A. S.	13.2	12.9	12.5	11.6	11.5	12.4	1.00
	12.5	12.9	13.4	13.3	10.1	11.2	
B. K.	14.7	14.1	13.4	13.4	12.5	13.8	0.76
	14.6	14.2	13.4	12.9	13.6	12.3	
S. B.	15.7	15.4	12.7	—	11.5	—	1.55
	14.2	15.3	14.9	—	13.9	—	
R. T.	16.8	17.3	16.3	—	20.8	—	1.56
	16.9	16.9	15.6	—	16.5	—	
M. F.	16.0	16.7	—	17.4	15.0	15.9	0.84
	16.0	16.8	—	17.7	15.7	17.0	
R. S.	17.3	18.3	16.1	—	14.9	16.0	1.61
	17.6	18.8	16.9	—	13.8	14.9	
L. H.	19.2	18.6	17.2	16.7	16.5	18.9	1.21
	19.5	17.8	16.7	17.2	16.5	19.5	
H. M.	19.5	19.1	18.7	19.5	16.8	19.0	0.92
	20.1	18.9	18.5	18.7	17.4	19.5	

c. In our management of myxedema, repeated serum PBI tests are done to observe the adequacy of dosage of the substitution thyroid hormone therapy. In the case of Mrs. M. A. an excessive number, 41 tests, were performed from January, 1954, until September, 1956.

Mrs. M. A. Before treatment the PBI values in this patient were *1.5 and 0.5* μg per cent; later, on sodium levothyroxin, 0.3 mg. by mouth daily, they were, successively, *7.2, 7.8, 6.4, 6.3, and 5.3* in a 3 month period; on 0.4 mg. in the next 2 months, they were *8.5 and 8.0;* 4 months later on the same dosage a report of *17.8* μg per cent was made because of *chemical mistake, clerical error, or the patient's ingestion of iodine.* Successive subsequent determinations on 0.2 mg. were *6.2 and 6.6,* on 0.4 mg. *8.0,* and for 4 months on 0.3 mg., *6.3, 6.0, 7.6, 7.2, 7.1, 7.5, and 7.8.* When the patient was changed to sodium dextrothyroxin, 0.5 mg. daily,

TABLE III. PBI VALUES OF THE SAME KNOWN CONTROL IN 25 CONSECUTIVE BATCHES (OCT. 1, 1958, TO JAN. 2, 1959; ESTABLISHED VALUE 5.2 TO 5.6 μG PER CENT)*

DATE	RESULT (μG%)	DATE	RESULT (μG%)	DATE	RESULT (μG%)	DATE	RESULT (μG%)	DATE	RESULT (μG%)
Oct.	5.4	Oct.	5.3	Oct.	5.4	Nov.	6.5	Dec.	5.2
Oct.	5.2	Oct.	5.6	Nov.	5.1	Nov.	5.2	Dec.	5.0
Oct.	5.4	Oct.	5.4	Nov.	5.2	Nov.	4.8	Dec.	6.0
Oct.	5.4	Oct.	5.3	Nov.	5.3	Dec.	5.3	Dec.	5.2
Oct.	5.5	Oct.	5.4	Nov.	5.5	Dec.	5.7	Jan.	5.5

*Normal control, Hyland Laboratories, Los Angeles, Calif.

TABLE IV.* THREE SUCCESSIVE SERUM PBI REPORTS IN 15 PATIENTS WITH MYXEDEMA BEFORE TREATMENT

PATIENT	FIRST (μg %)	SECOND (μg %)	THIRD (μg %)	PATIENT	FIRST (μg %)	SECOND (μg %)	THIRD (μg %)	PATIENT	FIRST (μg %)	SECOND (μg %)	THIRD (μg %)
R. Y.	1.2	1.7	1.8	D. C.	0.9	0.8	0.6	E. H.	1.1	1.2	0.9
R. T.	0.8	0.9	0.7	M. G.	1.2	2.0	2.0	E. H.	0.6	0.5	1.0
R. V.	1.0	1.0	0.7	M. W.	1.2	1.2	2.7	A. H.	1.1	1.4	1.5
A. S.	1.2	1.0	1.2	L. D.	2.1	2.0	3.2	F. H.	0.5	1.8	1.1
R. A.	1.7	1.7	1.4	L. E.	0.7	0.7	1.1	V. H.	1.3	1.8	1.3

*From the Chemistry Laboratory of the Los Angeles County Hospital. These patients were tested from 1953 to 1958. The repetitions were usually requested and performed within a 2 week period.

TABLE V. SEVENTEEN CONSECUTIVE DETERMINATIONS OF SERUM PBI IN DR. GEORGE S. ON T_3

DATE	PBI (μg %)	DATE	PBI (μg %)	DATE	PBI (μg %)	DATE	PBI (μg %)
7/13/53	2.5*	4/14/54	1.9	4/14/54	0.9	4/14/54	1.5
11/ 5/53	1.9	4/14/54	0.7	4/14/54	1.2	4/14/54	1.5
12/18/53	1.9	4/14/54	1.0	4/14/54	1.9	4/14/54	1.4
3/ 9/54	0.9	4/14/54	0.9	4/14/54	1.7	4/15/54	1.2
						4/19/56	1.3

*Suppression may not have been quite complete. Dr. S.'s normal serum PBI was about 4.5 μg per cent. From the clinical research laboratory (P.S.).

the PBI fell to *4.5 and 3.7,* on 1.0 mg. dextrothyroxin it became *6.5, 4.6, and 4.5;* on 0.2 mg. levothyroxin it was *6.4, and 5.5.* When 1 mg. of sodium levothyroxin was given by mouth, 7 serial PBI determinations within 24 hours showed the rise and fall of thyroxin in the blood. They were *5.5, 7.0, 7.4, 7.5, 6.4, 5.8, and 5.6.* When the same experiment was repeated later with sodium dextrothyroxin, 1 mg. orally, 8 values within 24 hours were *4.2, 5.4, 5.6, 5.7, 5.2, 4.6, 4.1, and 4.2.* In each of the last 2 experiments the final value is the same or within 0.1 μg of the initial value.

DISCUSSION

The effect of iodine-containing medication, food preparations, cosmetics, tooth paste, and dyes used for roentgenographic diagnosis is not reviewed. These substances may produce a true serum protein-bound iodine which is not derived from thyroxin. Nor is the fact reviewed that a postabsorptive basal state of the patient is not required to obtain true indication of thyroid hormone status. It should be emphasized that clear serum not mixed with hemolyzed erythrocytes is desirable; specimens sent by mail over the cells can be used with reliable results, but those with gross hemolysis are not determined. The red cells contain very little iodine so that the error produced by hemolysis is purely one of dilution. The thyroid hormone protein complex is remarkably stable; for example, as Raman and Beierwaltes[10] have shown, serum may be kept at room temperature in July in Ann Arbor, Michigan, for 4 weeks and be mailed to California without change in the PBI. Finally, the demonstration by Taurog and Chaikoff,[11] in 1948, that 98 per cent of the serum PBI is derived from circulating thyroxin should be recalled.

A large amount of additional data could be presented to demonstrate the clinical reliability of this test. Our subsequent experience has extended and substantiated the report of 1950.[6]

It is not generally appreciated that the cost to the patient of this test is less than that of the admittedly unreliable and nonspecific basal metabolic rate test. Furthermore, from the administrative viewpoint it is economical since one technician can perform from 75 to 100 determinations a week.

The determination of serum PBI is an exceedingly delicate test. Contamination with extraneous iodine and with unknown substances which inhibit the catalytic reduction of ceric ion in the color reaction are technical difficulties which can become quite serious unless scrupulous care is exercised. Accurate results, however, can be obtained by experienced analysts if each set of tests is adequately controlled by proper standards.

Accurate results obviously depend on experience, technical training, pure reagents, and internal controls.

SUMMARY

Duodecimal determinations of the protein-bound iodine (PBI) by means of the alkaline-incineration method were done on the sera of each of 15 hypothyroid patients. The range of PBI values in these patients was from 0.6 to 3.8 μg per cent. The average standard deviation of the determinations on each patient was $\pm$ 0.30 μg per cent. In 9 euthyroid patients, with PBI values ranging from 3.8 to 7.0 μg per cent, the average of such standard deviations was $\pm$ 0.47 μg per cent, and in 9 thyrotoxic patients, with PBI values from 12.0 to 21.6 μg per cent, was $\pm$ 1.05 μg per cent. In normal serum the 95 per cent confidence limits of determinations done in duplicate in these laboratories is approximately 0.5 μg per cent.

PBI values of the same known control serum done with 25 consecutive batches of specimens are tabulated.

Diagnostic PBI reports from 15 patients with myxedema, retested 3 times, are given.

Clinical observations of the PBI in 2 subjects treated, respectively, with triiodothyronin, and levo- and dextrothyroxin, for periods of 2 years are listed.

Such laboratory and clinical results indicate the satisfactory reliability of this procedure in the diagnosis and treatment of thyroid disease.

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THE TECHNIQUES AND INTERPRETATION OF PHAGE TYPING OF STAPHYLOCOCCI

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IN 1942 Fisk[1] described methods for the isolation and propagation of bacteriophages that are carried by lysogenic strains of *Staphylococcus aureus*, and utilized the selective action of such phages for the differentiation of cultures of staphylococci. Each phage was propagated by serial passage on a specific susceptible strain of *Staph. aureus* on agar to the point at which confluent lysis of the susceptible strain was obtained. The phage suspensions were freed from viable cocci by the addition of Zephiran in low concentration. Cultures of staphylococci to be identified by this method were inoculated on the surface of agar plates and one loopful of each of the test phages was "spotted" on the seeded area. After suitable incubation, differentiation of the cultures was accomplished by comparison of their patterns of susceptibility to the phages.

Wilson and Atkinson,[2] in 1945, modified Fisk's technique to permit the differentiation of staphylococci in a manner comparable to that which had been used successfully by Craigie and his associates[3] for phage typing of the typhoid bacilli. The phages were propagated either in young broth cultures of specific susceptible strains of staphylococci, or on the surface of agar plates according to Fisk's method, and were filtered through Seitz E. K. pads. An important modification introduced by Wilson and Atkinson was to employ the phages for typing at their test dilutions; this was the highest dilution of each phage that produced confluent lysis when spotted on its propagating strain on agar.

Using the phages and methods of Wilson and Atkinson, a phage typing service was established in 1946 in the Central Public Health Laboratory at Colindale, London, England, first under the direction of Dr. V. D. Allison and subsequently under that of Dr. R. E. O. Williams. Considerable experience in both the techniques and the interpretation of results of phage typing was gained from the studies in that laboratory. The publication in 1952 of an authoritative article by Williams and Rippon[4] has served as the point of reference for much of the work on phage typing of the staphylococci which is now being carried on in many laboratories throughout the world.

While the basic principles of the method are followed in all laboratories, the techniques vary in some details from one laboratory to another. It is probable that certain differences are permissible, provided that the objective

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is attained of producing phages of a potency and specificity which permit the reliable differentiation of cultures. However, as phage typing came to be more widely adopted it was recognized that some uniformity of methods would contribute to the confidence with which comparisons could be made of the observations reported by various laboratories. Such uniformity could be approached by some general agreement on basic techniques and criteria of interpretation. Accordingly, the Subcommittee on Phage Typing of Staphylococci, of the International Committee on Bacterial Nomenclature, was established during the Sixth International Congress for Microbiology in Rome in 1953. Although the members of the Committee have felt that rigid rules should not be established, they have formulated certain recommended basic methods of procedure (unpublished). Essentially, the methods follow those which have been evolved during the past several years in the Central Public Health Laboratory in London.[4-6]

We presented a report[7] on the bacteriophage typing of staphylococci in 1953 and described our methods, which were based upon those of Wilson and Atkinson. In the ensuing years certain modifications of technique were made and the methods were brought into general conformity with the recommendations of the International Committee. It is the purpose of this article to describe the methods in current use in this laboratory, which have been used successfully for the past several years, and to discuss the problem of the interpretation of the results of phage typing.

MATERIALS AND METHODS

Phages.—Twenty-three phages are used for routine typing. They include 21 basic phages that are recommended by the International Committee for routine use and 2 others that have been found useful in this laboratory. The phages fall into several broad groups, as follows:

Group	Phages
I	29, 52, 52A, 79, 80
II	3A, 3B, 3C, 55, 71
III	6, 7, 42E, 47, 53, 54, 73, 75, 77, 83
IV	42D
Miscellaneous	81, 187.

All of the above phages except 81 and 83 are basic phages. The broad groups, I, II, and III, are so numbered because it was found that the phages comprising each group generally exhibit some degree of specific action against the staphylococci belonging to the serologic groups 1, 2, and 3, respectively, that were established in 1939 by Cowan.[8] It should be pointed out that the correlation between the broad phage groups and the serologic groups is by no means complete.

All of the basic phages represent either phages that were used originally by Wilson and Atkinson or that were subsequently isolated in, or received at, the Central Public Health Laboratory at Colindale.* Phage 83 was isolated in this laboratory from a lysogenic strain of *Staph. aureus.*† It was originally designated by us as Phage VA4; the number 83 was assigned to it in 1958 by the International Committee. Phage 81 and its propagating strain were received from Dr. E. T. Bynoe,[9] in whose laboratory it was isolated.

Propagating Strains.—The specific propagating strains of *Staph. aureus* are identified by the same identifying numbers used for the phages, e.g., Strain PS 29 is used for the

*They were obtained through the courtesy of Dr. R. E. O. Williams.
†Supplied by Dr. M. L. Rakieten.

propagation of Phage 29, PS 3A for Phage 3A, and so on; Strain PS 52A/79 is used for the propagation of both Phage 52A and Phage 79.

The propagating strains are maintained on brain heart infusion agar slants in screw-capped tubes at room temperature, and are transferred weekly. A reserve stock of the propagating strains is stored on the same medium at room temperature in cork-stoppered, paraffined tubes and transferred at intervals of 6 to 12 months.

Propagation.—For all purposes of propagation, titration, and typing, trypticase soy agar and broth, pH 7.3,* are used. The method of propagation involves the growth of a mixture of phage and its specific propagating strain in soft agar which is layered over a solid agar base in Petri dishes; it was adapted from a method described by Swanstrom and Adams[10] for the propagation of *Escherichia coli* phages.

Two lots of trypticase soy agar are prepared. One contains 1.5 per cent agar for use as the base layer (and also for titration of the phages and for typing), and the other contains 0.7 per cent agar to be used for the propagating layer. For propagation, the 1.5 per cent agar is poured to a depth of about 8 mm. into Petri dishes 15 cm. in diameter, and the plates are dried at 30° C. for 18 to 24 hours.

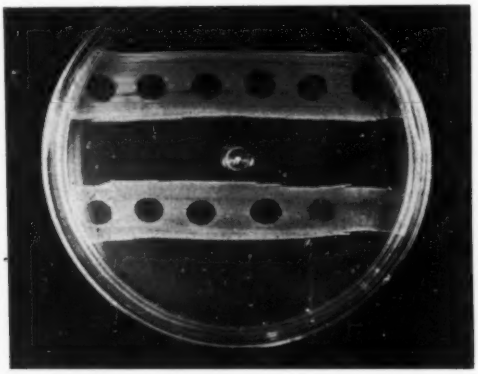

Fig. 1.—Titration of phages. Decimal dilutions range from 10^{-1} to 10^{-6}. Titrations of 2 phages are illustrated. In the upper one the titer is 10^{-5}; in the lower, the titer is 10^{-4}.

The growth from an 18 to 24 hour agar slant culture of the propagating strain is suspended in 2 ml. of broth. In each of 2 sterile tubes are placed 0.2 ml. of the staphylococcal suspension and 0.25 ml. of the stock phage, and to each tube is added 10 ml. of soft (0.7 per cent) agar which has been previously melted and cooled to about 45° C. The contents of each tube are mixed thoroughly and immediately poured over the base layer in Petri dishes. The plates are incubated for 18 to 24 hours at 30° C.

To harvest the phage, the soft agar layer is scraped up with a sterile glass rod and transferred to a sterile tube. A thin, alcohol flamed rod, bent at an angle of 90 degrees, and a powder funnel are convenient implements for removing the layer and transferring it to the tube. The surface of the base layer is washed off with 10 ml. of broth, which is added to the tube. The tubes are shaken vigorously to obtain a good suspension of the phage and are then centrifuged until the supernatant is clear. It is advisable to titrate the supernatant at this point, as described below, in order to determine the potency of the phage; if this appears to be low, 2 or 3 serial passages may then be carried out. When a satisfactory titer is obtained, the pooled supernatant from both tubes is filtered through a Seitz E. K. filter pad which has been thoroughly moistened with sterile broth; the filtrate is tested for sterility, and a final titration is set up. The filtrate represents the stock phage, which is stored at 4° C. in stoppered or screw-capped tubes.

*Supplied in dehydrated form by the Baltimore Biological Laboratories, Baltimore, Md.

Not infrequently a new stock of phage can be prepared directly from a potent, fluid preparation by a single passage as described above. In this laboratory it has rarely been necessary to carry a phage through more than 3 serial passages to produce a new stock of satisfactory potency. Several serial passages often are required when a new stock is made from a lyophilized preparation.

Alternatively, phage can be prepared on standard Petri dishes 10 cm. in diameter. Five or 6 plates are used for each phage to be prepared. The soft agar is used in a volume of 5 ml., and is inoculated with 0.1 ml. each of phage and staphylococcal suspension. Each plate is washed off with 5 ml. of broth and the washings are pooled for further processing.

Titration.—Serial decimal dilutions of the phage preparation, either supernatant or filtrate, are made in broth in a volume of 1 ml. ranging from 10^{-1} to 10^{-6}; a single 1 ml. pipette is used for the series. Trypticase soy agar plates that have been dried for 48 hours at 30° C. are employed. An area about 1.5 cm. in width is seeded across the surface of the agar from a 4 to 6 hour broth culture of the propagating strain, grown at 37° C.; a large loop, or a swab moistened in the culture, is used for the purpose. As soon as the inoculum

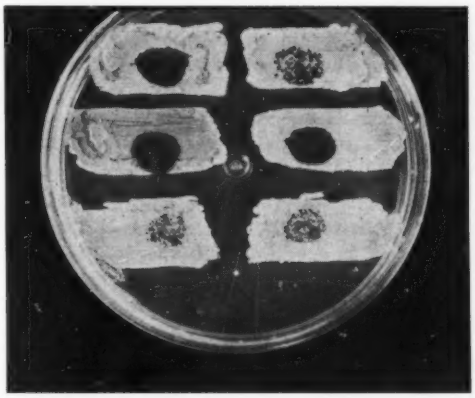

Fig. 2.—Spot checks of routine test dilutions. Checks of 6 phages are illustrated. Three show confluent lysis and are satisfactory for typing; 3 show less than confluent lysis and are unsatisfactory for typing.

has dried, a 4 mm. loopful of each phage dilution is deposited on the seeded area; the drops are spaced about 1.5 cm. apart. When the drops have dried, the plate is incubated at 30° C. for 18 to 24 hours. Four or 5 phages can be titrated on one 15 cm. plate; when a 10 cm. plate is used, adequate spacing of the dilutions permits the titration of only 2 phages.

The routine test dilution (R.T.D.) to be used for typing is the highest dilution of a phage that produces confluent lysis of the propagating strain on agar (Fig. 1). The R.T.D. differs from one phage to another; it often remains quite constant for any given phage, but it may vary up or down by one decimal dilution in successive lots of stock phage. Since the soft agar method of propagation was adopted in this laboratory, the majority of the phages have exhibited test dilutions of about 10^{-6}. None has dropped below 10^{-4} except on rare occasions, and then the titer could always be restored to 10^{-4} or better when the next lot of stock phage was prepared.

Routine Test Dilutions.—It is the practice in this laboratory to hold the entire series of dilutions used for titration in the refrigerator for 24 hours, when the plates are read and the R.T.D. is determined. Then the tube representing the R.T.D. is transferred to a rack reserved for the test dilutions that are used for routine typing. Should a larger volume of the test dilution be required than is customarily used for titration, it is easily prepared from the next stronger dilution of the titrating series. The potency of this preparation is tested by a spot check: an area about 2 cm. square on an agar plate is seeded from a 4 to

6 hour broth culture of the propagating strain, grown at 37° C.; one loopful of the test dilution is placed on the dried, seeded area, and the plate is incubated at 30° C. for 18 to 24 hours. A satisfactory preparation should produce confluent lysis.

The test dilutions are stored at 4° C., and are spot checked for potency at weekly intervals. The test dilutions are satisfactory for typing as long as they produce confluent lysis when spot checked on their propagating strains (Fig. 2). No preparation is used for typing unless it has shown good lysis within 7 days just prior to the typing. When the spot check shows less than confluent lysis, the preparation is discarded and a new test dilution is made. When this is required, it is advisable to retitrate the phage, since the opportunity is afforded of checking the stability of the stock preparation. Occasionally it is found that the potency of the stock has been slightly reduced and that the new test dilution should be one decimal dilution lower. The routine test dilutions have regularly remained satisfactory for typing for 2 to 6 weeks, and often for 8 or 10 weeks.

Phage Typing.—One standard 10 cm. Petri dish is used for each culture to be typed. The plates* are ruled in the form of a grid of thirty 11 mm. squares which serve as a convenient guide for spotting the test dilutions at regularly spaced intervals. The phages always are placed on the plates in an established sequence.

Trypticase soy agar plates for typing are poured and dried at 30° C. for 48 hours before use. Transfers to broth are made from fresh agar slant cultures of the staphylococci to be tested and incubated at 37° C. for 4 to 6 hours, when they exhibit a moderate, but well defined, turbidity. A sterile swab is moistened thoroughly in the broth culture, the excess fluid is removed by touching the inside of the tube, and the swab is streaked over the entire surface of the agar to obtain a uniform inoculum. On a properly prepared and dried plate the inoculum should dry within 5 minutes or less. The number or name of the culture is written above the top of the grid to identify it. When the inoculum is dry, a small drop of the test dilution of each phage is spotted in its appropriate square with a sterile Pasteur pipette, which has been drawn out to a fine tip. Care is taken to avoid touching the tip of the pipette to the agar. The drops of phage dry within a few minutes, and the plate is inverted and incubated at 30° C. for 18 to 24 hours.

Reading and Reporting.—The plates are read by the use of indirectly transmitted light against a dark background. While strong lysis is apparent by simple observation, weak reactions are best seen with the aid of a reading glass of moderate magnification.

Susceptibility to the phages is shown by the presence of areas of lysis at the site of one or more phages which range from isolated plaques to confluent lysis. While some strains are susceptible to only a single phage, lysis by several phages is encountered more commonly. The reactions occur in various combinations which are referred to as patterns of lysis, or phage patterns. The lytic reactions are recorded as follows:

Fifty plaques to confluent lysis	++
Twenty to 50 plaques	+
Less than 20 plaques	±
No lysis	−

All reactions which range from 50 plaques to confluent lysis are considered as strong reactions, and are regarded as significant to the composition of the pattern. Reactions of all degrees are entered in the laboratory record, but the *phage pattern* is reported in terms of those phages which produce significant lysis.

When no significant lysis is obtained with the routine test dilution of any phage in the series, typing of the culture is repeated with more concentrated phage preparations. For this purpose, the authors use a preparation of each phage that is 1,000 times stronger than its R.T.D., but in no case is a preparation used that is more concentrated than 10^{-1}. The results are recorded and interpreted according to the same principles that are followed when the routine test dilutions are used.

*The plates used in this laboratory were made for us by the Corning Glass Works, Corning, N. Y., and have a permanent grid etched on the bottom of the dish in "Lifetime Red." These plates are now available commercially.

Inhibition.—Certain potent phages exert an inhibitory effect when they are spotted on cultures of staphylococci. Inhibition is indicated either by an area of complete clearing entirely similar to that of confluent lysis or by an area of thinning or diminished density of growth at the site where the phage was deposited on the plate.[4, 6, 11] Clear areas of inhibition are usually produced only by undiluted phages or by preparations that are diluted not more than fivefold. It has been the experience in this laboratory that inhibition produced by higher dilutions usually is indicated by areas of diminished density of growth. They are not encountered frequently, and usually occur only when a previously nontypable culture is retyped with phages at 1,000 × R.T.D.

Williams and Rippon[4] state that the inhibiting agent passes through Seitz filters and Gradocol membranes. It appears to act only in the presence of phage, and it cannot be detected in preparations from which phage has been adsorbed on heat-killed staphylococci or neutralized by antiserum. It appears to differ from an autolytic agent described by Welsch and Salmon[12] but may be similar to a bacteriostatic agent described by Wahl and Josse-Goichet.[13]

Lytic Spectrum.—Each phage in the typing series exhibits a characteristic spectrum of activity against the strains of *Staph. aureus* that are used for propagation. The lytic spectrum of each phage remains quite constant and provides a measure of the purity of each new lot of phage.

Stock phages are spotted on all of the propagating strains and each phage is titrated against those strains which show any degree of lysis. The resulting titers are compared with the maximum titer shown by each phage against its specific propagating strain, and are graded according to their divergence from that titer.[4, 6, 14] Some slight changes in titer are permissible, but any radical change suggests that either a phage has undergone some variation in its host range or has become contaminated by another staphylococcal phage.

Similar information is obtained when all of the propagating strains are typed with the phages at R.T.D. The resulting patterns are also quite constant and should show, at the most, only minor variations from one lot of stock phage to the next. The phage patterns of the propagating strains obtained in this laboratory are shown in Table I. It should be noted that these patterns have been obtained consistently over a period of about 12 years, and compare favorably with the lytic spectrum described by Williams and his associates.

COMMENTS ON TECHNIQUE

Production of Phage.—That it shall support good growth of the staphylococci is an essential requirement of the culture medium used for propagation and for typing of the phages. This requirement appears to have been met by different media in various laboratories. Having selected a suitable culture medium, however, one should be on the alert for the possibility that an occasional lot of the medium may give relatively poor results.

In addition to the soft agar method of preparation described above, the phages can be propagated on surface cultures of the propagating strains on agar plates.[1, 2, 4, 7, 9] Usually the phage inoculum is spread over all but a small segment of the seeded area, which serves as a control of the staphylococcal growth. After suitable incubation the phage is harvested by washing off the lysed area with a small amount of sterile broth. Alternatively, the unlysed control area is removed with a scalpel and the remainder is frozen rapidly at a temperature below −10° C.; when frozen, the plate is held at room temperature and the fluid that is expressed during thawing is pipetted off. We have used both methods with satisfactory results. Surface propagation is especially useful when only a small amount of phage is available to initiate the preparation of a stock supply.

The majority of phages can be propagated successfully in broth, but it would appear that attention to certain details is essential.[2, 4, 15-19] Calcium is a necessary ingredient of the medium. Although at least traces of calcium are usually present in culture media, a deficiency of this element can be overcome by the addition of calcium chloride; a concentration of about 400 mcg. per milliliter is often used. The optimum ratio of cocci to phage for propagation in broth varies and can best be determined by preliminary trial. Broth cultures

are incubated at 37° C. for about 6 hours; mechanical shaking during incubation often enhances the production of phage. After incubation the culture is centrifuged and the supernatant is held in the refrigerator overnight, when lysis continues to take place.

The production of phage by the cellophane plate technique, sometimes used for the preparation of staphylococcal hemolysins, has been described.[20] The procedure is stated to be simpler and to require considerably shorter incubation than the soft agar method.

Preservation and Storage.—In the majority of laboratories, after harvesting and centrifugation, the phages are rendered free from bacteria by filtration, and are stored without preservative in the refrigerator at 4° C. Filtration involves the possible loss of some phage by adsorption. For this reason diatomaceous earth filters are not recommended. Seitz E. K. filter pads often are used; although there is some chance of adsorption, this can be minimized by moistening the filter pad thoroughly with sterile broth before use. Sintered glass filters,[21] Zsigmondy membrane filters,[9] and Gradocol membranes[4] have been found satisfactory. Others have avoided filtration and have relied upon a chemical agent to preserve the phage suspensions. Zephiran in low concentration,[1] thymol,[15, 17] and glycerol[21] have been employed.

TABLE I. LYTIC PATTERNS PRODUCED BY PHAGES AT R.T.D. ON PROPAGATING STRAINS

PROPAGATING STRAINS	PHAGES																						
	29	52	52A	79	80	3A	3B	3C	55	71	6	7	42E	47	53	54	73	75	77	83	42D	81	187
29	+																						
52		+																					
52A/79			+	+																			
80					+																	+	
3A						+																	
3B							+	±	±														
3C							+	+	±	+													
55					-		±	±	+	+													
71							±	v	±	+													
6											+			+	±	+		+		±			
7												+											
42E													+										
47														+				+		v			
53															+			+	±	+			
54														+	+	+		+	±	+			
73		±	±				v	v			v	±					+						
75																		+					
77																			+				
83											+			+	+				+	+			
42D																					+		
81					+																	+	
187																							+

For the purpose of this table only, the following symbols are used: + —occurs regularly in significant reactions, ± —usually occurs; sometimes weak reactions, and v —variable, usually weak reactions.

Lyophilization provides the ideal and probably the most satisfactory method of preservation and storage of stock phage preparations, but it must necessarily be confined to those laboratories that are adequately equipped for this method.[4, 9]

When stored in the refrigerator, the majority of undiluted, fluid phage preparations retain their potency for several months and usually remain viable, with some loss of their original potency, for well over a year. We have stored undiluted stock phages in sealed ampules at −10° C. Under these conditions all of a set of 24 phages remained viable for 2 years. The original titers of 15 (62.5 per cent) remained unchanged during this period. The titers of the others dropped by 2 or 3 decimal dilutions during the first year of storage; no further loss of titer occurred during the second year. Reserve stocks of the phages are maintained routinely at −10° C. in this laboratory.

Titration.—From published reports by other investigators and from several personal communications to the authors it is evident that the routine test dilutions of any given

phages differ to some extent in various laboratories. Assuming that a phage is of good initial potency, it seems apparent that these differences are due in part to differences in the technique of preparation of the dilutions for titration. It is well known that a higher titer may result when a single pipette is used for all dilutions in a series than when a new pipette is used for each successive dilution. In parallel titrations we have found that this difference may be as great as one hundredfold.

Typing.—The broth cultures used to inoculate plates for typing range in age from 4 to 6 hours[4, 7] to 18 to 24 hours.[9, 17, 21, 23] Studies by Williams and Rippon[4] indicate that no appreciable difference in the results is detectable when cultures of either age are used. When a large number of cultures must be typed within the space of the working day, considerable time is gained by using seed cultures 18 to 24 hours old. It is essential that the surface of the agar be dry, both to facilitate absorption of the inoculum and to prevent spreading of the drops of phage. It is advisable to apply the phages to the plates within a reasonably short time after they have been seeded; an interval longer than 1.5 hours would appear to reduce the intensity of the lytic reactions.[4]

An established, orderly arrangement of the phages on the plate aids in the reading and direct comparison of the patterns of lysis of a set of cultures. This is facilitated by ruling the plates with a glass marking, or a carborundum pencil, or by using a rubber stamp made in the form of a grid.[20, 22] In a few laboratories unruled plates are used, each plate being superimposed on a template which serves as a guide when the phages are applied.

Phages may be applied to the seeded plate by one of several methods: fine capillary tubes,[15, 23] a Pasteur pipette which has been drawn out to a fine tip,[4, 7] or a tuberculin syringe fitted with a 27 gauge needle.[21] Mechanization has recently entered this field, for a semiautomatic, multiple loop device which applies all of the phages at one time has been described by Tarr.[24] This device is now in use in some laboratories. Another apparatus which applies all of the phages simultaneously to the plate has been devised by Goldberg,[25] of the Bureau of Laboratories of the New York City Department of Health.

The plates may be incubated for 4 to 6 hours at 37° C. and then held at room temperature overnight before reading,[9, 15, 18] or they may be incubated continuously at 30° C. for 18 to 24 hours.[4, 21] We have used both methods and prefer the latter. Room temperature may fluctuate during an overnight period of incubation, and varies with the season of the year. Continuous incubation at 30° C. appears to result in more consistent and satisfactory lysis; it avoids the necessity, often under the pressure of the daily schedule, of waiting to transfer the plates from the incubator to the laboratory bench after 4 to 6 hours of incubation at 37° C. It is not advisable to incubate the plates continuously at 37° C. for 18 to 24 hours, for heavy growth of the cocci tends to obscure phage activity.

INTERPRETATION

It is possible to demonstrate with reasonable accuracy the probable relationship or lack of relationship of cultures of coagulase-positive staphylococci by means of phage typing. The method is particularly suited to the examination of sets of cultures from sources which suggest that the cultures possess some degree of identity; it is thus a useful adjunct to the epidemiologic investigation of staphylococcal disease. Basically, identical cultures exhibit identical or closely similar phage patterns; distinct differences in their patterns indicate that the cultures are not related.

It is not difficult to determine that cultures are identical when they are lysed by the same combination of phages. It will be remembered that all degrees of lysis from about 50 plaques to confluent lysis are considered as strong reactions and enter into the formation of the phage pattern. Consequently, 2 patterns that are composed of the same combination of phages are regarded as identical although the lysis produced by the several phages which make up the

pattern may cover the entire range of strong reactions. Weak or moderate reactions of less than 50 plaques are not considered to be of significance, especially when they are not a part of the pattern common to 2 cultures. However, weight can be given to an occasional weak reaction when it occurs together with significant reactions as part of an identical pattern that is exhibited by a series of presumably related cultures.

It is not difficult to determine that cultures are unrelated when they exhibit distinctly different phage patterns. The chief problems of interpretation are presented by presumably related cultures which exhibit patterns that are so nearly alike as to suggest that the cultures are identical, but which, nevertheless, do not show complete correspondence. For example, 2 presumably related cultures may show nearly identical phage patterns which differ only by the presence in either pattern of 1 or 2 significant reactions that are absent from the other. When such differences occur it becomes necessary to decide whether the divergence of the patterns is sufficient to indicate that the cultures are distinct or whether the resemblance is close enough to suggest that the cultures probably are related.

Small differences in the patterns of related cultures, whether they are differences in the degree of lysis or qualitative differences in the composition of the pattern, frequently result from the variations that must be expected in any bacterial population. In a detailed study of these variations, Williams and Rippon[4] have shown that the chance that variation will occur in the patterns of related cultures increases with the remoteness of the individual cultures from their presumed common source. Thus the basic phage pattern of a culture is reproducible with only minor variations in replicate cultures of the parent strain or in cultures isolated at the same time from several sites in one individual. Greater deviations from the basic pattern, sometimes involving the gain or loss of a strong reaction, may be seen in a set of cultures isolated during an episode of hospital cross infection, especially if it is prolonged. Such variations do not affect the basic stability of the phage pattern, nor do they detract from the validity of the results of phage typing. They must, however, be taken into account in order to evaluate the significance of the apparent similarities or differences of the observed phage patterns.

While they sometimes appear to be confusing, these variations do not present insurmountable problems to the interpretation of the phage patterns. A guide to interpretation is offered by Williams and his associates.[4, 6] When all cultures of a presumably related set are typed on the same day, it may be assumed that the cultures are probably identical if their patterns do not differ by more than one strong reaction. Cultures are regarded as different when the pattern of 1 contains 2 or more strong reactions that are completely absent from the other. This criterion has been adopted as a useful guide in many laboratories with generally satisfactory results. However, it is not inflexible and circumstances sometimes require its modification. As mentioned above, weak reactions may be of significance under certain conditions. Occasionally differences of 2 or more significant reactions in the patterns of 2 cultures can be interpreted rather liberally; this can be done only when knowledge of the source and

history of the cultures strongly suggest that they probably are related. Only experience can determine those instances in which such a liberal interpretation is justified. Phage typing is a tool which complements other data necessary to the epidemiologic investigation of staphylococcal infection. As such, the results must be interpreted in the light of other known facts concerning the source, date of isolation, and presumed relationships of cultures to be submitted to phage typing. It seems reasonable to suggest that all pertinent information about the cultures should be available to the bacteriologist. This need not introduce an undesirable subjective factor into the interpretation of results. It can be of distinct help in the determination of probable relationships of cultures which exhibit moderately disagreeing patterns, or, occasionally, patterns that vary beyond the usually accepted criteria.

When cultures are not lysed or when they show only weak or moderate reactions when typed with the routine test dilutions, they are retyped with more concentrated phage preparations. The selection of the most suitable concentration for retyping is a problem which has not been adequately solved, but many laboratories now use preparations 1,000 times stronger that the routine test dilutions of the phages. In any event, dilutions stronger than 1:10 should not be employed because of the probable occurrence of reactions of inhibition. The results of typing with the more concentrated preparations are interpreted according to the principles mentioned above, but caution must be used because the stronger preparations tend to produce rather broad, probably nonspecific, lytic patterns, often complicated by the presence of weak or moderate reactions. Wahl and Fouace[11, 15] suggest that instead of typing at R.T.D. and retesting at 1,000 × R.T.D., a single typing be done with the phages at about 100 × R.T.D. There appear to be no published reports of the efficiency of this concentration; Anderson and Williams[6] suggest that it may contribute to the complexity of patterns of cultures which can be identified just as readily when typed at R.T.D.

Very occasionally cultures have been encountered in this laboratory which were not lysed when typed at R.T.D., but which exhibited a pattern consisting only of reactions of inhibition when retested at 1,000 × R.T.D. Such reactions appeared as areas of less dense growth at the site of the phages involved. Sets of repeated cultures from single lesions in a few patients consistently have shown such reactions in patterns characteristic for the set. It would appear that on rare occasions a consistent reaction pattern of inhibition may provide the only clue to the probable identity of presumably related cultures.

Some cultures of coagulase-positive staphylococci are untypable at either R.T.D. or at 1,000 × R.T.D. The proportion of untypable cultures varies with different sets of cultures and may be as high as 20 per cent. We can confirm the observation of Williams and Rippon[4] that untypable coagulase-positive staphylococci are encountered more commonly in cultures from the nose than from lesions. It is conceivable, but not proved, that some cultures are insusceptible to the phages because of certain inherent metabolic differences. It is also possible that the standard series of typing phages lacks a phage to which the untypable cultures might be susceptible. In a few instances cultures which could not be identified by the basic series of phages have been differentiated by means of

a new phage isolated by the cross-culture method of Fisk,[1] or by adaptation of one of the basic phages to a new staphylococcal host. Often the usefulness of such a phage is confined to the characterization of cultures isolated during a strictly localized outbreak of infection. Phages 80 and 81, however, which were produced by adaptation of basic phages to new hosts, have proved to be of wide usefulness.[9, 26]

In a few laboratories it is the practice to retest untypable cultures with certain phages which have less activity than those of the basic series. These phages are grouped into pools (there appears to be no interference of one by another), and a culture that is lysed by a pool is then retested with the individual phages which comprise the pool. It sometimes is possible to differentiate a few additional cultures in this way. It was reported, for example, by Anderson and Williams[6] that about 65 per cent of cultures were typable at R.T.D., and an additional 27 per cent at 1,000 × R.T.D.; only 8 per cent were not typable at these dilutions. Of these latter, from 10 to 50 per cent could be typed with the extra phages.

The nature of the phages and of the method requires that careful attention be given to the established procedures. It is a fundamental principle that the phages should be used for typing at their proper test dilutions. The potency of the test dilution of each phage should be determined by spot check against its propagating strain at least once a week, and any dilution of reduced potency should be replaced by a suitable preparation. Undetected alterations of potency of the test dilutions can result in patterns that are subject to erroneous interpretation. As mentioned above, there is no evidence that phage preparations stronger than the test dilutions provide significantly better results in primary typing. Our experience and that of others indicate that when stronger preparations are used for primary typing the patterns sometimes can be confusing and misleading. Preparations stronger than the test dilutions should be reserved for the examination of culture which either are not lysed or give less than significant reactions when typed with the routine test dilutions. It cannot be assumed that any stock preparation of phage retains its original potency indefinitely when stored either as a fluid preparation at 4° C. or in the lyophilized state. Regardless of its original potency, every phage must be titrated to determine its proper test dilution when removed from stock or when reconstituted from a lyophilized preparation. Each phage is characterized by a spectrum of activity against the several propagating strains.[4, 14] A determination of the spectrum of each new lot of phage provides a control on its purity; this can be done readily by spotting the phage at its R.T.D. on all propagating strains of the series. While minor variations in the spectrum may occur from one lot of phage to the next, any great deviation from the usual host range suggests that a phage may have undergone mutation, or even become contaminated, and is unsuitable for typing. Preferably, all cultures of a presumably related set should be typed on the same day to avoid chance variations due to differing experimental conditions. When this cannot be done, the patterns obtained in the 2 series of typings are interpreted with due regard to the possible occurrence of some variation.

The practice of routine screening of cultures isolated in the diagnostic laboratory against only 2 or 3 phages to detect the presence of staphylococci of a single, presumably epidemic type would appear to be unwise. It is true that strains of certain types, notably 80/81, have been shown to be responsible for many outbreaks of staphylococcal disease, and there appears to be little question that staphylococci of this type often possess an extraordinary capacity for infectivity. However, we feel that the emphasis which has been given to this type has led to the erroneous assumption that other types have no epidemic significance. Williams[27] has shown convincingly that a variety of types can be responsible for an epidemic under specific conditions. This suggests that to screen cultures routinely for only one type is to risk missing another strain or strains that have assumed epidemic status in the hospital or local area where this practice is carried out.

Phage typing is a rather expensive procedure in terms of time, personnel, and equipment, and is therefore not readily adaptable to the average hospital laboratory. While this seems unfortunate in view of the prevalence of staphylococcal infections, it may be pointed out that many cultures of staphylococci isolated in the diagnostic laboratory do not need to be phage typed. With the exception of certain controlled research applications of basic value, phage typing of the staphylococci should be limited to the study of specific outbreaks of infection where, within the context of other available epidemiologic data, the probable relationships of cultures can be determined. The usefulness of the method in this field has been adequately demonstrated. Preferably, cultures of probable epidemiologic significance should be examined in central laboratories that are staffed with trained personnel who are competent not only to perform the tests but to interpret the results with due regard to the variables that may be encountered. Typing of the single culture, unrelated to other known infections, provides no significant diagnostic information and merely adds to the burden of the phage typing laboratory.

SUMMARY

The techniques of propagation and titration of staphylococcal phages, tests for their control, and the method of phage typing as practiced in our laboratory are described.

Modifications of some details of these procedures, as practiced in other laboratories, are described and discussed.

The problems of interpretation of the results of phage typing are discussed and our views and suggestions are outlined.

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